THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 39, Issue of September 30, pp. 24245–24251, 1994
Printed in U.S.A.

Type II Regulatory Subunit (RII) of the cAMP-dependent Protein Kinase Interaction with A-kinase Anchor Proteins Requires Isoleucines 3 and 5*

(Received for publication, April 18, 1994, and in revised form, June 24, 1994)

Zachary E. Hausken‡, Vincent M. Coghlan‡, Cheryl A. Schafer Hastings§, Erwin M. Reimann§, and John D. Scott‡¶

From the ‡Vollum Institute, Portland, Oregon 97201-3098 and the §Medical College of Ohio, Toledo, Ohio 43699-0008

Compartmentalization of the type II cAMP-dependent protein kinase is maintained by association of the regulatory subunit (RII) with A-Kinase Anchor Proteins (AKAPs). In previous studies (Scott, J. D., Stofko, R. E., McDonald, J. R., Comer, J. D., Vitalis, E. A., and Mangili J. (1990) *J. Biol. Chem.* 265, 21561–21566) we have shown that dimerization of RIIα was required for interaction with the cytoskeletal component microtubule-associated protein 2. In this report we show that the localization and dimerization domains of RIIα are contained within the first thirty residues of each RII protomer. RII des-5 (an amino-terminal deletion mutant lacking residues 1–5) was unable to bind AKAPs but retained the ability to dimerize. RIIα I3A,I5A (a mutant where isoleucines 3 and 5 were replaced with alanine) was unable to bind a variety of AKAPs. Mutation of both isoleucines decreased AKAP binding without affecting dimerization, cAMP binding, or the overall secondary structure of the protein. Measurement of RIIα I3A,I5A interaction with the human thyroid AKAP, Ht 31, by two independent methods suggests that mutation of isoleucines 3 and 5 decreases affinity by at least 6-fold. Therefore, we propose that two isoleucine side chains on each RII protomer are principle sites of contact with the conserved amphipathic helix binding domain on AKAPs.

The actions of many hormones proceed through common signal transduction pathways that generate the intracellular second messenger cAMP (1). The predominant action of cAMP is to activate a cAMP-dependent protein kinase (PKA)[1] by binding to the regulatory subunit (R) dimer of the holoenzyme thereby releasing the catalytic (C) subunit (2). Free C subunit potentiates hormonal responses by phosphorylating substrate proteins surrounding the site of kinase activation. Although the general principles of the cAMP-signaling pathway are well understood (reviewed in Refs. 3 and 4), it remains unclear how individual hormones influence PKA to phosphorylate specific proteins. One hypothesis that accounts for these observations is that hormones selectively activate compartmentalized PKA pools leading to the preferential phosphorylation of the appropriate substrates. For this to occur PKA must be maintained in specific cellular compartments where it is co-localized with target substrates. Subcellular fractionation and immunolocalization studies have demonstrated that PKA is attached to certain subcellular structures (5–7), and it has been shown that the type II regulatory subunit (RII) is localized through interaction with specific A-kinase anchor proteins (AKAPs) (8–11).

Although there is little overall sequence homology among AKAPs, a consensus RII-binding domain has been defined (12–18). Each anchoring protein contains a region of approximately 20 residues responsible for RII binding that is likely to form an amphipathic helix (15–20). Mutations which disrupt the secondary structure within the putative helices of the human thyroid anchoring proteins, Ht 31 and AKAP 79, destroy RII interaction (15, 17). Furthermore, peptides spanning the helix region of Ht 31 bind RIIα or the type II holoenzyme with high affinity (K_D ~4 nM) and are potent inhibitors of PKA anchoring (16). More recently, these "anchoring inhibitor peptides" have been used as reagents to disrupt the localization of the type II PKA *in vivo* (21).

The focus of this report is to define the determinants on RII responsible for binding to the AKAP. Previous work using the cytoskeletal component MAP 2 as a model has shown that the first 50 residues of RIIβ are required for AKAP binding (22), whereas other studies have shown that dimerization is a prerequisite for anchoring (23). In this report we identify specific amino acids within the first 5 residues in each RIIα protomer that are essential for anchoring. Mutation of two isoleucine residues, conserved in all RII isoforms, decreases AKAP binding without disrupting dimerization or the secondary structure of RIIα.

EXPERIMENTAL PROCEDURES

Construction and Expression of Truncated RIIα Proteins—Truncated forms encoding the first 40, 35, and 30 residues of RIIα were constructed by PCR using a Coy TempCycler and pET RIIα as a template. Each PCR reaction used a common 5′ primer (100 pmol) encoding an *Nde*I site surrounding the start codon (CCCCCATATGGGCCACATCCAG), whereas unique 3′ primers were synthesized to provide the appropriate coding region of RIIα and a stop codon. Unique 3′ primers (100 pmol) encoding a *Bam*HI site past the stop codon (CGCGCGGATCCTCACAGGCGTGTGAAG), (CGCGCGGATCCTACTCCACCGCGAAG) and (CGCGCGGATCCTAGACGAGGTCGGGC) were used for 30 rounds of PCR (0.5-min denaturation 95 °C, 1.5-min anneal 40 °C, 2-min extension 72 °C) using *Taq* polymerase. PCR products were digested with *Nde*I and *Bam*HI to excise 120-, 105-, and 90-base pair fragments encoding the first 40, 35, and 30 residues of RIIα, respectively. *Nde*I-*Bam*HI cut DNA fragments were inserted into the HisTag™ bacterial expression vector pET16d (Novagen) and transfected into competent *Escherichia coli* BL 21 (DE 3) cells for expression of the truncated proteins. PCR products were sequenced to confirm the correct sequence. Nucleotide sequencing was performed by the method of Sanger *et al.* (24).

* This work was supported by National Institutes of Health Grant DK 44238 (to J. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Vollum Institute, 3181 S. W. Sam Jackson Park Rd., Portland, OR 97201-3098. Tel.: 503-494-4652; Fax: 503-494-2285.

[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchor protein; MAP, microtubule-associated protein; PCR, polymerase chain reaction; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochlorine; HPLC, high performance liquid chromatography.

One liter of LB broth containing 100 μg/ml of ampicillin was inoculated with an overnight culture of *E. coli* transformed with a pET RII1–30 HisTag. The cells were grown at 37 °C to an $OD_{550\ nm}$ of 0.3 in a shaking incubator. Maximal expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were grown for an additional 2.5 h to allow accumulation of the recombinant protein. The culture was centrifuged at 8,000 rpm for 8 min at 4 °C and resuspended in a buffer of 20 mM Tris, pH 7.9, 5 mM imidazole, 500 mM NaCl, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochlorine (AEBSF). HisTag proteins (10 histidines at the amino terminus of each recombinant protein) were purified by affinity chromatography on a nickel-charged His-bind™ metal chelation resin and were eluted by washing with buffers containing 400 mM imidazole.

Construction of RIIα Des-5 and RIIα Des-10—Deleted RII forms were produced by replacing a 42-base pair *Nco*I-*Pst*I segment at the 5′ end of the coding region with pairs of oligonucleotide linkers to eliminate 5 or 10 amino acids from the amino terminus. In two separate reactions the expression plasmid pET11d RII (5 μg) was digested with *Pvu*I and *Nco*I to obtain a 4,700-base pair fragment, containing the majority of pET11d (reaction 1) or *Pst*I and *Pvu*I to obtain a 2,400-base pair fragment, which contained the RIIα coding region downstream from the *Pst*I site (reaction 2). Plasmids were reconstructed by three-way ligation with equimolar amounts of DNA inserts (reactions 1 and 2), and a 95-fold molar excess of the appropriate oligonucleotide linkers. The oligonucleotide linkers (ATGGGCCCGGGGCTCACGGAGCTCCTGCA) and (GGAGCTCCGTGAGCCCCGGGCC) were used to reconstruct RIIα des-5 and (CATGGAGCTCCTGCA) and (GGAGCTCC) to reconstruct RIIα des-10. A silent G to C mutation was introduced into the oligonucleotide pairs to generate a *Sac*I site to facilitate identification of mutated plasmids. The coding regions of RIIα des-5 and RIIα des-10 were removed from pET11d by digestion with *Nco*I and *Eco*RI, inserted into pET9d, and both plasmids were transfected into competent *E. coli* BL 21 (DE 3) cells. The 5′ end of the coding region was sequenced to confirm the appropriate changes.

Site-directed Mutagenesis—All mutants were created in the 5′ end of the RIIα coding region by modification to the PCR method Scharf *et al.* (25) using a COY TempCycler and pET RIIα as a template. Unique 5′ PCR primers (GATATACCATGGGCCACGCCGCGGCCCCGCCGGGGC) encompassing the *Nco*I/start site and containing the appropriate base mismatches to create mutations between residues 3–5 were annealed to the murine RIIα cDNA. Each PCR reaction utilized a common 3′ primer (TCTCGGGGTATTGTA). Point mutations in RIIα sequence were introduced at Ile^3 (ATC), Gln^4(CAG) and Ile^5 (ATC). Thirty cycles of PCR amplification (0.5-min denaturation at 95 °C, 1.5-min anneal at 40 °C, 2-min extension at 72 °C) were performed with 100 pmol of primer and using *Taq* polymerase to create a 644-base pair fragment. DNA fragments were digested with *Nco*I and *Sal*I to excise 90-base pair inserts encompassing the mutations and inserted into the bacterial expression vector pET11d containing the remainder of the RIIα coding region and transfected into competent *E. coli* BL 21 (DE 3) cells. The coding region was sequenced to confirm the mutation and sequence.

All mutant and deleted RIIα forms were expressed in *E. coli* and purified as described (23) with the following minor modifications. DNase I treatment of the bacterial cell lysates was omitted, and the proteinase inhibitor AEBSF (ICN Biomedicals) was included in the protein storage buffer at a final concentration of 0.1 mM. Concentration of purified proteins was achieved by ultrafiltration through 10,000 NMWL regenerated cellulose (Amicon).

RII Overlay—The RII overlay binding assay was performed according to the method of Lohmann *et al.* (26) with modifications to the method of Bregman *et al.* (12). Recombinant RIIα was phosphorylated using recombinant catalytic subunit as described (27). All overlays were performed in solutions containing 250,000 cpm of radiolabeled RIIa or mutants. As additional controls, overlays were performed in the presence of 0.3 μM anchoring inhibitor peptide (DLIEEAAVSRIVDAVIEQVKAAGAY) which competes for RII/AKAP interaction or a control peptide (DLIEEAAVSRPVDAVIEQVKAAGAY) which is unable to block binding. Detection of truncated RII forms was by modified Western blots performed by the method of Towbin *et al.* (28). Quantitative overlays were performed by essentially the same methods except Ht 31 (at concentrations ranging from 0.2–100 ng) was absorbed onto nitrocellulose using a Hybri-slot Manifold (Life Technologies, Inc.). Binding was detected by autoradiography and was measured by densitometry after scanning into a computer and analyzed by the National Institutes of Health image 1.55 program.

Band Shift Analysis—The ability of RII mutants to dimerize or form complexes with AKAPs was assessed by nondenaturing gel electrophoresis using the previously described technique (16). The RII dimer or the RII·AKAP complex migrates slower than the monomeric RII and could be easily distinguished

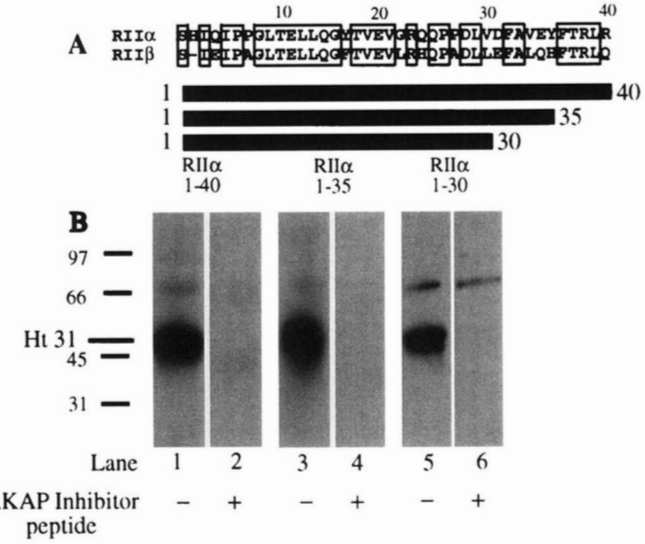

FIG. 1. **The amino-terminal region of RIIα bind AKAPs.** *A*, the amino acid sequences of murine RIIα and RIIβ are shown. The *boxed regions* indicate sequence identity between both RII isoforms. *B*, the AKAP binding properties of truncated RII forms was assessed by the solid-phase overlay assay. A representative example of four experiments is presented. Purified Ht 31 (5 μg) was separated by gel electrophoresis on a 10% SDS-polyacrylamide gel. After electrophoresis, the protein was electrophoretically transferred to Immobilon for analysis by overlay binding assay using truncated RIIα proteins (20 μg) as probes: *lanes 1* and *2*, RIIα Δ1–40; *lanes 3* and *4*, RIIα Δ1–35; *lanes 5* and *6*, RIIα Δ1–30. Molecular mass markers were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Immobilized RIIα forms were detected immunochemically with polyclonal anti RII antisera (28). Filters in *lanes 2, 4*, and *6* were incubated in the presence of the AKAP inhibitor peptide.

Circular Dichroism Analysis—Circular Dichroism spectra were recorded using a Jasco 500A spectropolarimeter. Solutions of RIIα and RIIα mutant proteins (5–32 μM) were dialyzed into 50 mM potassium phosphate buffer, pH 7.0. Measurements were performed at 25 °C with identical data acquisition parameters, and spectra were normalized at 192 nm.

Equilibrium Dialysis—Binding of RIIα and mutant proteins (20 nM) to the anchoring inhibitor peptide, Ht 31 493–515, was measured by equilibrium dialysis using our previously described method (16). All experiments were performed with a final concentration of ^{125}I-labeled peptide at 14 nM of specific activity of 5×10^6 cpm/nmol.

RESULTS

Localization Domain Is Contained within the First 30 Residues of RIIα—In previous studies the MAP 2 binding region was shown to require sites within the first 79 amino acids of RIIα (23) and the first 50 residues of RIIβ (22). Comparison of the murine RIIα and RIIβ sequences revealed a high degree of homology (65% identity) in the first 40 residues (Fig. 1*A*), whereas both sequences diverge from residues 40 to 75 (29). Based upon the assumption that conserved structure often reflects conserved function, we concluded that the AKAP-binding region must be located between residues 1–40. To test this hypothesis a family of truncated RIIα forms were expressed in *E. coli* using the bacterial expression vector pET16d and the HisTag™ purification system. Truncated RII forms containing residues 1–40, 1–35, and 1–30 of murine RIIα bound the AKAP Ht 31, as assessed by the RII overlay procedure (Fig. 1*B*). Detection of RII was achieved with polyclonal antiserum raised to recombinant RIIα, and binding was blocked in the presence of 0.3 μM AKAP inhibitor peptide (16) Ht 31 493–515 (Fig. 1*B*). These results indicate that the AKAP-binding site is located within the first 30 residues of RIIα.

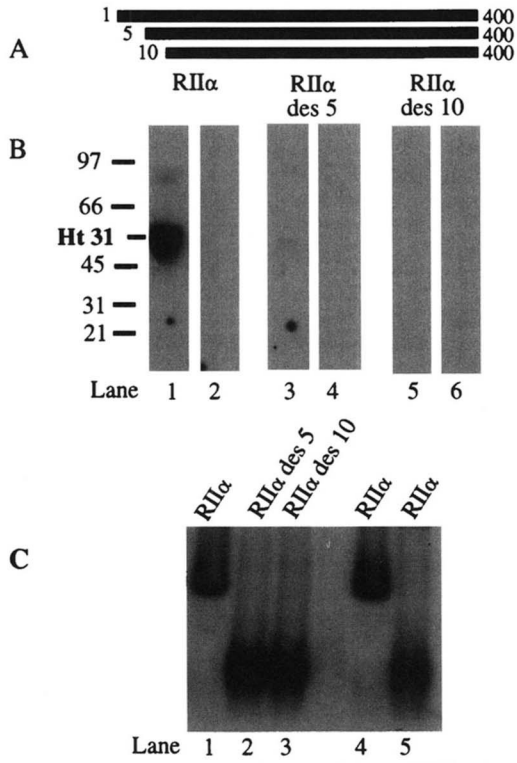

FIG. 2. **Removal of residues 1–5 abolished RII/AKAP interaction.** *A*, RIIα and deletion mutants lacking the first 5 and 10 residues of each RII protomer were analyzed for AKAP interaction. *B*, purified Ht 31 (5 μg) was separated by gel electrophoresis on a 10% SDS-polyacrylamide gel. After electrophoresis, the protein was electrophoretically transferred to Immobilon for analysis by solid-phase overlay binding assay (26) using radiolabeled RIIα (*lanes 1* and *2*), RIIα des-5 (*lanes 3* and *4*), or RIIα des-10 (*lanes 5* and *6*) as probes. The specific activity of the probes were: RIIα, 3.2×10^5 cpm/nmol; RIIα des-5, 2.9×10^5 cpm/nmol, and RIIα des-10, 3.4×10^5 cpm/nmol. Molecular mass markers were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase, (31 kDa), and soybean trypsin inhibitor (21 kDa). Filters in *lanes 2, 4*, and *6* were incubated in the presence of Ht 31 493–515 peptide which is a potent competitive inhibitor of RII/AKAP interaction (16). *C*, AKAP binding in solution was analyzed by the band shift analysis using ^{32}P-radiolabeled AKAP 95 as a probe. RIIα and deletion mutants (1 μM) were incubated with AKAP 95 (1 μM), of specific activity 4.5 cpm/nmol, under the conditions described in Ref. 15, and separated on a 6% polyacrylamide gel: *lane 1*, RIIα; *lane 2*, RIIα des-5; *lane 3*, RIIα des-10; *lane 4*, RIIα + control AKAP inhibitor peptide, and *lane 5*, RIIα + AKAP inhibitor peptide. Detection was by autoradiography.

Deletion of the Amino-terminal Residues 1–10—We have shown previously that deletion of the first 30 residues of RIIα prevents dimerization (23). On the basis of this observation and the results described above, we postulated that the dimerization contact sites and the AKAP-binding sites could reside between residues 1 and 30 of each RII protomer. To test this hypothesis, deletion mutants lacking the first 5 or 10 amino acids of RIIα were constructed (Fig. 2*A*), and the recombinant proteins were expressed in *E. coli* and purified by affinity chromatography on cAMP-Sepharose. Both deleted RII forms failed to bind immobilized Ht 31 as assessed by the overlay procedure (Fig. 2*B*) and were unable to complex in solution with a nuclear matrix-associated anchoring protein, called AKAP 95, as assessed by the band shift analysis technique (Fig. 2*C*). In contrast, full-length RIIα bound either Ht 31 or AKAP 95 when used as a control in these experiments (Fig. 2, *B* and *C*), and as expected, binding was blocked in the presence of the 0.3 μM AKAP inhibitor peptide (Fig. 2, *B* and *C*).

Since neither RIIα des-5 nor RIIα des-10 bound AKAPs, it was imperative to establish whether the deleted RIIα forms could dimerize. RIIα des-5 was a dimeric protein as assessed by

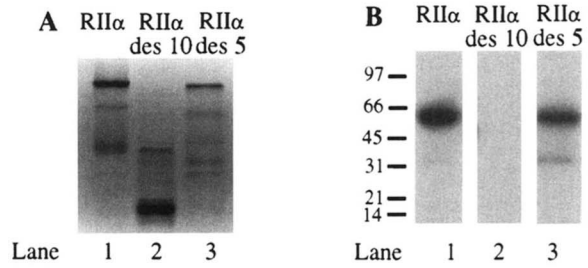

FIG. 3. **Deletion of residues 1–5 does not prevent RII dimerization.** RIIα and deletion mutants lacking the first 5 and 10 residues of each RII protomer were analyzed for the ability to dimerize. *A*, purified proteins (2 μg) were separated by electrophoresis under nondenaturing conditions on a 6% polyacrylamide gel: *lane 1*, RIIα; *lane 2*, RIIα des-10; and *lane 3*, RIIα des-5. Detection of proteins was by staining with Fast stain™ (Zion Research). *B*, solid-phase dimer formation was assessed by the overlay assay (26). Full-length proteins RIIα (*lane 1*), RIIα des-10 (*lane 2*), and RIIα des-5 (*lane 3*) (1 μg) were separated by gel electrophoresis on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to Immobilon for analysis by overlay assay using radiolabeled RIIα (of specific activity 3.2×10^5 cpm/nmol) as a probe. Detection was by autoradiography. Molecular mass markers were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa).

several criteria: the protein migrated with an apparent mobility similar to wild type RIIα when separated by nondenaturing gel electrophoresis (Fig. 3*A*, *lane 3*); the immobilized protein bound radiolabeled RIIα as assessed by a modified overlay assay (Fig. 3*B*, *lane 3*); and the protein eluted with an apparent mobility as full-length RIIα on a Sep-Pak 300 HPLC gel-filtration column (data not shown). In contrast, RIIα des-10 was monomeric: the purified protein migrating with a distinct mobility when separated by nondenaturing gel electrophoresis (Fig. 3*A*, *lane 2*); the immobilized protein was unable to bind radiolabeled RIIα in the modified overlay assay (Fig. 3*B*, *lane 2*); and the protein eluted with a molecular weight consistent with a 45-kDa monomer on Sep-Pak 300 HPLC gel-filtration column. Full-length RIIα was used as a control for these experiments and was dimeric under both experimental conditions (Fig. 3*A*, *lane 1*, and *B*, *lane 1*). Collectively, these findings suggest that RIIα des-5 is dimeric, whereas RIIα des-10 is monomeric, which indicates that determinants for AKAP binding are located within the first 5 residues of each RII protomer.

Disruption of AKAP Interaction by Mutagenesis of Residues 3–5 of RII α—On the basis of the experiments described above, residues 1–5 of RIIα (Ser-His-Ile-Gln-Ile-) were identified as potential sites for interaction with AKAPs. By process of elimination, only residues 3–5 were considered as likely determinants for AKAP binding. Recombinant RIIα retains wild-type AKAP binding activity, although it contains a glycine at position 1 instead of serine. Histidine 2 was considered to be an unlikely determinant for AKAP binding also, as it is not present in RIIβ (Fig. 1*A*). Therefore, each side chain between residues 3 and 5 was systematically substituted for alanine (Fig. 4*A*), and the AKAP binding properties of the mutant proteins were compared with full-length RIIα. Each RIIα protein was used in overlay assays at approximately the same specific activity (7.2 to 6.1×10^5 cpm/nmol). Single site mutants of either the isoleucine 3 or the isoleucine 5 (RIIα I3A and RIIα I5A) exhibited diminished binding to a variety of AKAPs by the overlay assay (Fig. 4B, *lanes 2* and *4*) when compared with full-length RIIα (Fig. 4*B*, *lane 1*). More pronounced effects were observed in RIIα mutants lacking both isoleucine side chains. The double mutant RIIα I3A,I5A (Fig. 4*B*, *lane 5*) and the triple mutant RIIα 3–5 AAA (Fig. 4*B*, *lane 6*) were unable to bind AKAPs assessed by the overlay assay. In contrast, mutation of glutamate 4 had no apparent effect on solid-phase AKAP binding

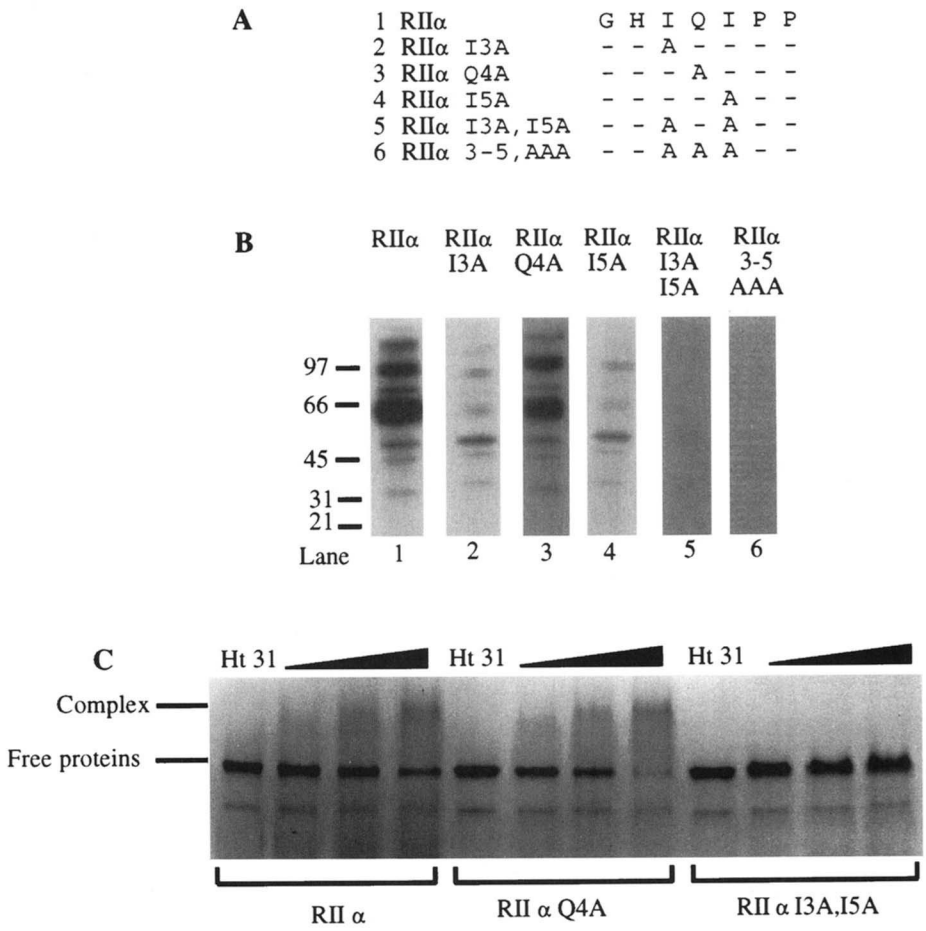

FIG. 4. **Mutation of isoleucines 3 and 5 impairs AKAP binding.** *A*, a family of mutants were created at sites between residues 3 and 5 on each RII protomer by a PCR mutagenesis method (24). Amino acids are indicated by the one-letter code. *B*, the AKAP-binding properties of RIIα mutants was assessed by the solid-phase overlay assay. Total cell proteins from a mouse insulin secreting β-pancreatic cell line (50 μg) were separated by gel electrophoresis on a 5–20% gradient SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to Immobilon for analysis by overlay binding assay using phosphorylated RIIα mutants as probes: *lane 1*, RIIα; *lane 2*, RIIα I3A; *lane 3*, RIIα Q4A; *lane 4*, RIIα I5A; *lane 5*, RIIα I3A,I5A; *lane 6*, RIIα 3–5 AAA. Each overlay was performed with a probe of approximately the same specific activity (7.2 to 6.1 × 10^5 cpm/nmol). Detection was by autoradiography. Molecular mass markers were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin, (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). *C*, AKAP binding in solution was analyzed by the band shift analysis using Ht 31 as a probe. Selected RIIα mutants (1 μM) were incubated with aliquots of Ht 31 over a range of concentrations (0.33, 0.66, and 1 μM) under the conditions described in Ref. 16. The free and complexed proteins were separated on a 6% polyacrylamide gel: *left panel*, RIIα; *middle panel*, RIIα Q4A; and *right panel*, RIIα I3A,I5A. Detection was by protein staining with Fast stain™ (Zion Research).

(Fig. 4*B, lane 3*), suggesting that isoleucines 3 and 5 are anchoring determinants. A similar pattern was obtained when AKAP binding in solution was assessed by band shift analysis (Fig. 4*C*); RIIα Q4A essentially retained wild-type binding properties when compared with full-length RIIα, whereas the double mutant RIIα I3A,I5A was unable to complex with Ht 31 over the same range of concentrations (Fig. 4*C*). Likewise, no binding to Ht 31 was obtained with the triple mutant RIIα 3–5 AAA (data not shown).

Because mutation of isoleucines 3 and 5 diminished AKAP binding, it was important to establish that these changes did not disrupt the overall conformation of the R subunits or prevent dimerization. The structural integrity of each mutant RII protein was confirmed by several criteria. The secondary structure of each point mutant was assessed by circular dichroism and compared to recombinant RIIα (Fig. 5*A*). CD spectra for each mutant were similar to those obtained for wild type protein, suggesting that mutation of residues 3–5 minimally perturbs the conformation of the RIIα subunits. Furthermore, each purified mutant protein (Fig. 5*B*) retains the ability to dimerize with RII when immobilized on nitrocellulose as assessed by the overlay assay (Fig. 5*C*) and dimerizes on the basis of migration on nondenaturing gels (Fig. 5*D*). Moreover, each protein binds cAMP and was purified by affinity chromatography on cAMP-Sepharose and is eluted as dimers with an apparent molecular mass of 110,00 kDa on a Sep-Pak 300 HPLC gel-filtration column and autophosphorylates with similar kinetics as wild type RIIα (data not shown). Combined, these data strongly suggest that mutation of isoleucines 3 or 5 in RIIα decreases the AKAP binding without altering the overall conformation of the protein or affecting other RII functions.

Quantitation of AKAP Binding to RIIα and Mutants—Overlay and band shift analysis techniques suggested that mutation of isoleucines 3 and 5 decreased the affinity for AKAPs. Therefore, binding of the various RIIα mutants to Ht 31 protein and the anchoring inhibitor peptide, Ht 31 493–515, was measured in order to determine changes in AKAP binding affinity. Quantitative overlays were performed using each RIIα protein at approximately the same specific activity (2.16 to 1.5 × 10^5 cpm/nmol), and binding to Ht 31 was measured by densitometry over a range of concentrations (3–1,000 ng). Single site mutation of either isoleucine 3 or 5 decreased binding to Ht 31 (Fig. 6*A*) and binding of the double and triple mutants was markedly decreased (Fig. 6*A*). In contrast, mutation of glutamine 4 to alanine had a modest effect (Fig. 6*A*). Fig. 6*B* compares the binding properties of RIIα and mutants at a single concentration of Ht 31 (250 ng). Mutation of either isoleucine 3 or 5 decreased binding by 39 and 60%, respectively, whereas mutation of glutamine 4 had no effect (Fig. 6*B*). Binding of the double and triple mutants were decreased by 81 and 83%, respectively (Fig. 6*A*).

In order to measure AKAP interaction by an alternative method, binding of each RIIα mutant to the Ht 31 493–515 peptides was determined by equilibrium dialysis. All studies were performed with 20 nM protein, a concentration where wild type RIIα binds 66% of the peptide (Table I). Equilibrium dialysis measurements confirmed the results obtained by the quantitative overlay technique: binding of single site mutants at either isoleucine 3 or 5 was decreased 39.2% ± 8.3 (n = 6) and 38.8% ± 8 (n = 6), respectively (Table I); and binding of the double and triple mutants were decreased 84.2% ± 4.7 (n = 6) and 79.1% ± 6.8 (n = 6), respectively. Binding of the RIIα Q4A was essentially the same as wild type (92% ± 8.4, n = 6). Col-

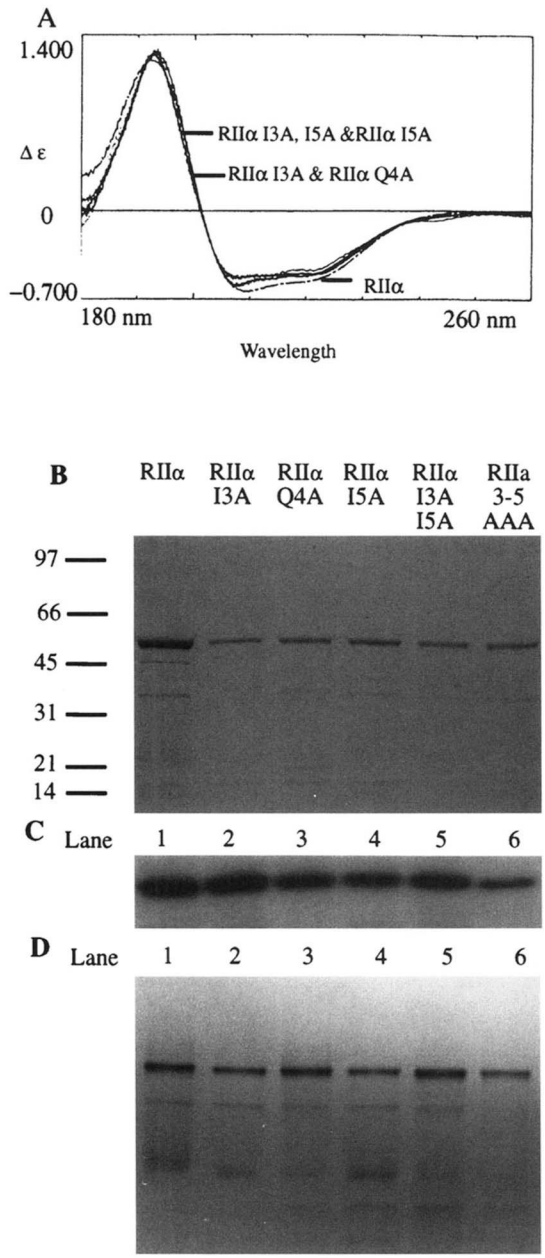

FIG. 5. **Characterization of RIIα mutants.** *A*, each RIIα mutant was purified by affinity chromatography on cAMP-Sepharose as described under "Experimental Procedures." *A*, circular dichroism spectra were measured as described under "Experimental Procedures." Spectra for individual mutants are indicated. *B*, the purity of each RII mutant was assessed by electrophoresis on a 10% SDS-polyacrylamide gel: *lane 1*, RIIα; *lane 2*, RIIα I3A; *lane 3*, RIIα Q4A; *lane 4*, RIIα I5A; *lane 5*, RIIα I3A,I5A; *lane 6*, RIIα 3–5 AAA. Detection was by protein staining with Fast stain™. Molecular mass markers were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). *C*, solid-phase dimer formation was assessed by the overlay assay. Full-length RIIα and mutants (1 μg) were separated by gel electrophoresis on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to Immobilon for analysis by overlay binding assay using radiolabeled RIIα as a probe (specific activity: 5.8×10^5 cpm/nmol). Detection was by autoradiography. *D*, dimerization of RIIα mutants (4 μg) was assessed by electrophoresis under nondenaturing conditions on a 6% polyacrylamide gel. Detection of proteins was by staining with Fast stain™ (Zion Research).

lectively, these results suggest that mutation of either isoleucine decreases AKAP binding by a factor of 2–3-fold, but mutation of both residues decreases binding by a factor of up to 6-fold (at this concentration of RII).

DISCUSSION

A growing body of evidence suggests that R of the cAMP-dependent protein kinase are composed of functionally distinct domains (30, 31). Limited proteolysis, chemical modification, and recombinant DNA techniques have been used to identify individual domains in both RI and RII responsible for homodimer formation, inhibition of the C subunit, and cAMP binding (32–35). Although both R subunit types contain domains that participate in each of these functions, an additional property of RII is that it can be localized to particular subcellular compartments through association with AKAPs. In this report we show that the localization domain is contained within the first 30 residues of each RII protomer. We have proposed previously that this region forms the dimerization domain and is required to orient the side chains in each RII protomer that form the AKAP-binding site (23). We can now extend this observation to conclude that AKAP binding and dimer formation are mediated through distinct side chains. This was shown by deletion of RIIα residues 1–5 which abolished interaction with anchoring proteins without any apparent effect upon dimerization. This result indicates that determinants for localization reside in the extreme amino terminus of each RII protomer and that side chains which participate in dimer contact are located further downstream. Despite the apparent segregation of localization and dimerization determinants, it is clear that other factors contribute to high affinity interaction with AKAPs. Luo *et al.* (22) have shown residues 1–25 of RIIβ are unable to bind AKAP 75 or MAP 2 when fused at the carboxyl terminus of a 43-kDa *Trp* E gene product. Furthermore, our own work has shown that fusion of residues 1–30 of RIIα to endonexin permits dimerization but prevents anchoring (23). Presumably, fusion to a carrier protein either disrupts the orientation of the AKAP-binding site or prevents dimerization. Recent evidence suggests that phosphorylation of RIIβ may also affect its ability to bind AKAPs and hence its subcellular localization. Keryer *et al.* (35) have shown that *in vitro* phosphorylation of RIIβ at threonine 69 by the cell cycle regulator kinase $p34^{cdc2}$ decreases affinity for MAP 2. Although phosphorylation of the RIIβ is an elegant means of regulating its subcellular location, it may not be a generalized mechanism, as the threonine 69 site is not present in any of the RIIα isoforms.

Our site-directed mutagenesis studies suggest that isoleucines at positions 3 and 5 in each RII protomer are positive determinants for AKAP binding, whereas the intervening residue, glutamine 4, is neutral. As can be seen in Fig. 1*A*, the amino-terminal regions of both RII isoforms are highly conserved with both isoleucines invariant in all species with the exception of the yeast RII homolog, *bcy* 1 (36), which is unable to bind AKAPs.[2] In contrast, glutamine 4 is not completely conserved and glutamate occupies this position in RIIβ. The presence of a negatively charged side chain in RIIβ could permit the formation of additional interactions with the anchoring protein and may account for some of the reported differences in the affinity of RIIβ for selected AKAPs (9 37).

Mutational analysis shows that replacement of either isoleucine 3 or isoleucine 5 had less of an effect on AKAP binding than substitution of both side chains. In keeping with our hypothesis that dimerization is an absolute requirement for AKAP interaction, single-site mutations correspond to a net loss of two isoleucine side chains from the RII dimer, whereas four hydrophobic side chains are missing in the double mutant. These findings suggest that branched chain aliphatic side chains interact with the anchoring protein. In complimentary studies we have shown that amphipathic helices form the RII-binding

[2] V. M. Coghlan and J. D. Scott, unpublished observation.

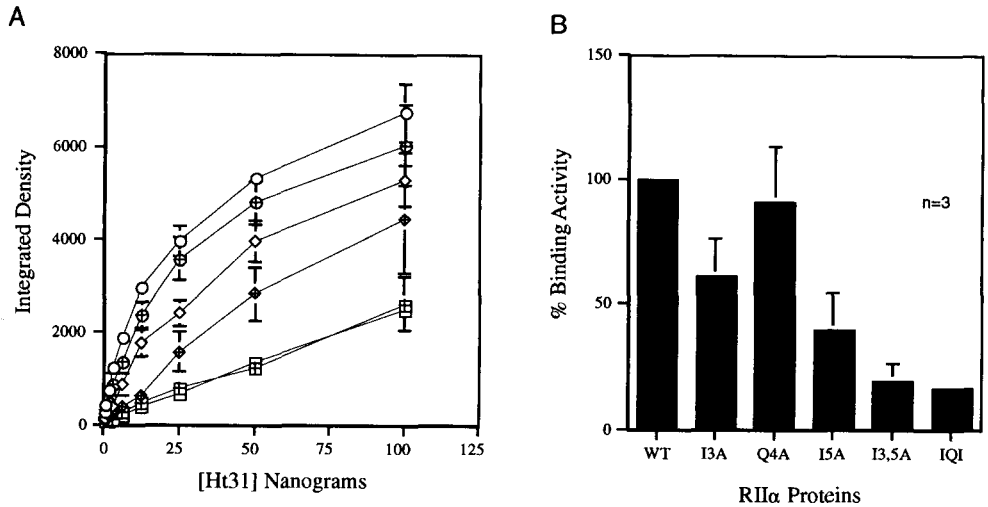

FIG. 6. **Quantitation of AKAP binding to RIIα mutants.** The binding of RIIα and mutants to a recombinant fragment of the human thyroid anchoring protein Ht 31 was measured by a quantitative overlay procedure. Aliquots of purified Ht 31 protein, ranging from 0.2 to 100 ng, were immobilized onto nitrocellulose filters. Individual filters were probed with excess RIIα or mutants as probes (specific activities ranging from 2.1 × 10^5 cpm/nmol to 1.5 × 10^5 cpm/nmol). Detection of binding was by autoradiography. *A*, quantitation of binding over a range of Ht 31 concentrations was measured by densitometry of the autoradiographs. Signals were normalized for the specific activities of each probe. Binding curves for individual protein probes from three experiments are: ○, RIIα; ◇, RIIα I3A; ⊕, RIIα Q4A; ◆, RIIα I5A; □, RIIα I3A,I5A; and ⊞, RIIα IQI,AAA. *B*, the degree of Ht 31 binding obtained at a single concentration of protein (25 ng) is presented as a percent binding compared with wild-type RIIα. The data are collected from three separate experiments.

TABLE I
Equilibrium dialysis of RIIα proteins

Binding of each RIIα mutant to the Ht 31 493–515 (14 nM) was measured by equilibrium dialysis. All studies were performed with 20 nM of protein, a concentration where RIIα binds 66% of the Ht 31 peptide. The binding properties of each RIIα mutant are presented as the ratio of bound peptide to total peptide (column 1) and as the percent of binding compared with that for wild type RIIα (column 2). The number of times each experiment was performed is indicated.

Protein (20 nM)	Bound/total	Binding
		%
RIIα (n = 7)	65.9 ± 3.0	100 ± 4.6
RIIα I3A (n = 6)	40.1 ± 5.5	60.8 ± 8.3
RIIα Q4A (n = 6)	60.8 ± 5.5	92.3 ± 8.4
RIIα I5A (n = 6)	40.3 ± 5.3	61.2 ± 8.0
RIIα I3A,I5A (n = 6)	10.4 ± 3.1	15.8 ± 4.7
RIIα IQI,AAA (n = 6)	13.8 ± 4.5	20.9 ± 6.8

sites in several AKAPs (15–18). Thus, isoleucines 3 and 5 on each RII protomer may interact with the hydrophobic face of the amphipathic helix binding region of AKAPs. This hypothesis is consistent with our findings that agents which disrupt hydrophobic interactions such as low concentrations of acetonitrile abolish RII/AKAP interaction[3] and is supported by previous findings (38) that demonstrate that substitution of hydrophobic residues in the amphipathic helix region of AKAP 75 decreases affinity for RIIβ.

A schematic diagram showing possible topologies for RII/AKAP interaction is presented in Fig. 7. A parallel orientation would cluster the essential isoleucine residues and form a hydrophobic face on RII that could form hydrophobic contacts with a corresponding region provided by the amphipathic helix of the AKAP (Fig. 7*A*). Alternatively, an antiparallel orientation would place a pair of isoleucine residues at either end of the dimerization surface, implying that additional points of contact reside between residues 5 and 30 of each RII protomer (Fig. 7*B*). Equilibrium dialysis data suggest that mutation of isoleucines 3 and 5 cause at least a 6-fold decrease in binding. This implies that either substitution of alanine still permits hydrophobic interaction with the AKAP or, alternatively, additional

[3] D. W. Carr and J. D. Scott, unpublished observation.

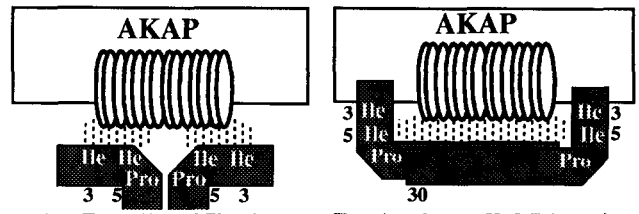

FIG. 7. **Models for RII/AKAP interaction.** A schematic diagram depicting possible topologies of RII/AKAP interaction. Two possible alignments are presented: a parallel orientation (*A*) or antiparallel orientation (*B*). *Shaded area* represents potential sites for hydrophobic interactions between RII and the amphipathic helix on the AKAP.

sites of contact reside within the RII dimer. Residues 11–25 on each RII protomer have a high probability for formation of β-sheet, which could comprise the dimerization surface. Structural studies with a biotinylated RII 1–30 peptide were unsuccessful as the peptide was insoluble in all of the aqueous buffers required for binding studies. Therefore, we cannot definitively distinguish between either dimerization model until the three-dimensional structure of RII has been solved. On the other hand, *in vitro* -linking studies have indicated that the RI dimer adopts anti parallel orientation through the formation of interchain disulfide bonds between Cys^{16} and Cys^{35} on each protomer (39, 40). Although it seems logical to propose that both types of R subunit dimer would have similar topologies, there is no sequence similarity between the dimerization domains of RI and RII (30, 31).

In conclusion, we propose that branched chain hydrophobic amino acids at positions 3 and 5 on RII maintain interaction with an amphipathic helix on the AKAP. Quantitative analysis suggests that the binding affinity of RIIα is decreased by up to 10-fold when both isoleucines are replaced with neutral amino acids. Although it is clear that dimerization is required for RII/AKAP interaction, distinct side chains participate in both functions. An additional determinant may be proline 6, which is conserved in all RII forms and may function to orient isoleucines 3 and 5 as the rigid properties of imino linkage, could perturb the RII peptide backbone (Fig. 7). Mutagenesis studies have provided RIIα proteins which have diminished ability to

bind a variety AKAPs *in vitro*. Future studies are planned to establish whether introduction of RIIα I3A,I5A or RIIα des-5 into cells can increase the soluble pool of type II PKA and alter certain cellular responses to cAMP.

Acknowledgments—We thank J. Randy MacDonald and Dr. Hans Peter Bachinger for assistance with CD analysis, Dr. Linda B. Lester for providing proteins from a mouse insulin secreting β-pancreatic cell line, and colleagues in the Vollum Institute for valuable discussions.

REFERENCES

1. Sutherland, E. W. (1972) *Science* **171,** 401–408
2. Scott, J. D. (1991) *Pharmacol. Ther.* **50,** 123–145
3. Beebe, S. J. & Corbin, J. D. (1986) *Enzyme (Basel)* **17,** 43–111
4. Taylor, S. S., Buechler, J. A. & Yonemoto, W. (1990) *Annu. Rev. Biochem.* **59,** 971–1005
5. Corbin, J. D., Keely, S. L. & Park, C. R. (1975) *J. Biol. Chem.* **250,** 218–225
6. Rubin, C. S., Erlichman, J. & Rosen, O. M. (1972) *J. Biol. Chem.* **247,** 6135–6139
7. Rubin, C. S., Rangel-Aldao, R., Sarkar, D., Erlichman, J. & Fleischer, N. (1979) *J. Biol. Chem.* **254,** 3797–3805
8. Theurkauf, W. E. & Vallee, R. B. (1982) *J. Biol. Chem.* **257,** 3284–3290
9. Leiser, M., Rubin, C. S. & Erlichman, J. (1986) *J. Biol. Chem.* **261,** 1904–1908
10. Sarkar, D., Erlichman, J. & Rubin, C. S. (1984) *J. Biol. Chem.* **259,** 9840–9846
11. Scott, J. D. & McCartney, S. (1994) *Mol. Endocrinol.* **13,** 5–11
12. Bregman, D. B., Bhattacharyya, N. & Rubin, C. S. (1989) *J. Biol. Chem.* **264,** 4648–4656
13. Bregman, D. B., Hirsch, A. H. & Rubin, C. S. (1991) *J. Biol. Chem.* **266,** 7202–7213
14. Hirsch, A. H., Glantz, S. B., Li, Y., You, Y. & Rubin, C. S. (1992) *J. Biol. Chem.* **267,** 2131–2134
15. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. S., Brennan, R. G. & Scott, J. D. (1991) *J. Biol. Chem.* **266,** 14188–14192
16. Carr, D. W., Hausken, Z. E., Fraser, I. D. C., Stofko-Hahn, R. E. & Scott, J. D. (1992) *J. Biol. Chem.* **267,** 13376–13382
17. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Cone, R. D. & Scott, J. D. (1992) *J. Biol. Chem.* **267,** 16816–16823
18. Coghlan, V. M., Langeberg, L. K., Fernandez, A., Lamb, N. J. C. & Scott, J. D. (1994) *J. Biol. Chem.* **269,** 7658–7665
19. Rubino, H. M., Dammerman, M., Shafit-Zagardo, B. & Erlichman, J. (1989) *Neuron* **3,** 631–638
20. Obar, R. A., Dingus, J., Bayley, H. & Vallee, R. B. (1989) *Neuron* **3,** 639–645
21. Rosenmund, C., Carr, D. W., Bergeson, S. E., Scott, J. D. & Westbrook, G. L. (1994) *Nature* **368,** 853–856
22. Luo, Z., Shafit-Zagardo, B. & Erlichman, J. (1990) *J. Biol. Chem.* **265,** 21804–21810
23. Scott, J. D., Stofko, R. E., McDonald, J. R., Comer, J. D., Vitalis, E. A. & Mangili, J. (1990) *J. Biol. Chem.* **265,** 21561–21566
24. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
25. Scharf, S. J., Horn, G. T. & Erlich, H. A. (1986) *Science* **233,** 1076–1078
26. Lohmann, S. M., DeCamili, P., Einig, I. & Walter, U. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6723–6727
27. Scott, J. D., Fischer, E. H., Demaille, J. G. & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4379–4383
28. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
29. Scott, J. D., Glaccum, M. B., Zoller, M. J., Uhler, M. D., Helfman, D. M., McKnight, G. S. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5192–5196
30. Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A. & Titani, K. (1984) *Biochemistry* **23,** 4200–4206
31. Titani, K., Sasagawa, T., Ericsson, L. H., Kumar, S., Smith, S. B., Krebs, E. G. & Walsh, K. A. (1984) *Biochemistry* **23,** 4193–4199
32. Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M. & McCarthy, D. (1978) *J. Biol. Chem.* **253,** 3997–4003
33. Reimann, E. M. (1986) *Biochemistry* **25,** 119–125
34. Ringheim, G. E. & Taylor, S. S. (1990) *J. Biol. Chem.* **265,** 4800–4808
35. Keryer, G., Luo, Z., Cavadore, J. C., Erlichman, J. & Bornens, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5418–5422
36. Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurtwitz, M., Krebs, E. G. & Wigler, M. (1987) *Mol. Cell. Biol.* **7,** 1371–1377
37. Carr, D. W., DeManno, D. A., Atwood, A., Hunzicker-Dunn, M. & Scott, J. D. (1993) *J. Biol. Chem.* **268,** 20729–20732
38. Glantz, S. B., Li, Y. & Rubin, C. S. (1993) *J. Biol. Chem.* **268,** 12796–12804
39. First, E. A. & Taylor, S. S. (1984) *J. Biol. Chem.* **259,** 4011–4014
40. Bubis, J., Vedvick, T. S. & Taylor, S. S. (1987) *J. Biol. Chem.* **262,** 14961–14966