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Mutational Analysis of the A-Kinase Anchoring Protein (AKAP)-binding Site on RII

CLASSIFICATION OF SIDE CHAIN DETERMINANTS FOR ANCHORING AND ISOFORM SELECTIVE ASSOCIATION WITH AKAPs*

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Compartmentalization of the type II cAMP-dependent protein kinase is conferred by interaction of the regulatory subunit (RII) with A-Kinase Anchoring Proteins (AKAPs). The AKAP-binding site involves amino-terminal residues on each RII protomer and is formed through dimerization. A site-directed mutagenesis strategy was utilized to assess the contribution of individual residues in either RII isoform, RIIα or RIIβ, for interaction with various anchoring proteins. Substitution of long-chain or bulky hydrophobic groups (leucines or phenylalanines) for isoleucines at positions 3 and 5 in RIIα decreased AKAP-binding up to 24 ± 3 (n = 8)-fold, whereas introduction of valines had minimal effects. Replacement with hydrophilic residues (serine or asparigine) at both positions abolished AKAP binding. Mutation of proline 6 in RIIα reduced binding for four AKAPs (Ht31, MAP2, AKAP79, and AKAP95) from 2.3 to 20-fold (n = 4) whereas introduction of an additional proline at position 6 in RIIβ increased or conferred binding toward these anchoring proteins. Therefore, we conclude that β-branched side chains at positions 3 and 5 are favored determinants for AKAP-binding and prolines at positions 6 and 7 increase or stabilize RIIα interaction with selected anchoring proteins.

Hormone action is a complicated and dynamic process that requires the efficient transmission of information from the extracellular environment to precise intracellular sites. The speed and precision of these events is a fascinating topic which has engaged researchers for more than three decades. As a result there has been a tremendous emphasis on the elucidation of signal transduction pathways. The work of numerous investigators have shown that many hormone-stimulated signaling cascades emanate from transmembrane receptors at the plasma membrane and proceed through intermediary G-proteins to promote the stimulation of adenylyl cyclase (1, 2). The net effect of this transduction process is the generation of the diffusible second messenger molecule cAMP which binds and activates the cAMP-dependent protein kinase (PKA)[1] (3). The kinase is activated by the release of two catalytic subunits (C) from the regulatory (R) subunit-cAMP complex. The free catalytic subunits are then able to phosphorylate a variety of substrate proteins on serine or threonine which contain the consensus sequence Arg-Arg-Xaa-Ser/Thr-Xaa or Lys/Arg-Arg-Xaa-Xaa-Ser/Thr-Xaa (4). An array of PKA isozymes are expressed in mammalian cells, and genes encoding three C subunits (Cα, Cβ, and Cγ) and four R subunits (RIα, RIβ, RIIα, and RIIβ) have been identified (reviewed in Ref. 4). Two holoenzyme subtypes called type I and type II are formed by the combination of RI or RII with the C subunits (5, 6).

Although many hormones use parallel pathways to activate PKA, some measure of specificity must be cryptically built into each signaling cascade to ensure that the correct pool of kinase becomes active in the right place and at the right time (7). It is now evident that additional regulatory mechanisms are in place to restrict the subcellular location of active enzyme. In fact, up to 75% of type II PKA is localized to various intracellular sites through association of the regulatory subunit (RII) with protein kinase A anchoring proteins, called AKAPs (reviewed by Rubin (8) and Scott and McCartney (9)). An additional level of selectivity may exist in certain cell types as both RII isoforms, RIIα and RIIβ, seem to preferentially associate with different anchoring proteins (10). For example, follicle-stimulating hormone treatment of rat granulosa cells increases expression of an 80-kDa AKAP which preferentially associates with RIIα (11), whereas phosphorylation of bovine RIIβ by cyclin B/p34^{cdc2} kinase decreases affinity for the microtubule-associated protein 2 (MAP2) (12).

Our previous studies have shown that RII dimerization is an absolute prerequisite for anchoring and that the AKAP-binding site resided in the amino-terminal 79 residues of RII (13). This model was later refined by Erlichman and colleagues (14), who concluded that sites within the first 50 residues of RIIβ were sufficient for anchoring. More definitive studies demonstrated that the localization and dimerization determinants were contained in the first 30 residues of each RII protomer (15, 16). In this report we have used a variety of binding assays to monitor the relative affinities of RIIα, RIIβ, and mutants for four AKAPs: Ht31, MAP2, AKAP79, and AKAP95. These studies have allowed us to detect subtle differences in the AKAP-binding preferences of RIIα and RIIβ and ascertain some of the side chain determinants on RII which direct the subcellular location of PKA. Another consequence of this work has been the generation of RIIα mutants that are unable to associate with AKAPs *in vitro* or *in situ*. It is anticipated that the detailed analysis of these mutants will contribute to our understanding of RII-AKAP interactions and provide new reagents to probe the function of PKA anchoring inside cells.

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[1] The abbreviations used are: PKA, protein kinase A; MAP2, microtubule-associated protein 2; AKAP, A-kinase anchoring protein.

MATERIALS AND METHODS

Site-directed Mutagenesis of RII—Mutations were created in the 5′ end of the RIIα coding region by modification of the polymerase chain reaction method of Scharf and colleagues (17). Manipulations were performed on a COY TempCycler using RIIα as a template. Degenerate oligonucleotide primers GATATA*CCATGG*GCCAC**ATC**CCG**ATC**CCGCCGGGGC which encompassed the initiation codon and changed codons 3 and 5 to leucine (**CTG**), phenylalanine (**TTC**), valine (**GTC**), serine (A**G**C), or asparigine (A**AC**) were used to generate an initial set of RIIα mutants. Substitution of prolines 6 or 7 with alanine (**G**C**G**) utilized a different primers GATATA*CCATGG*GCCACATCGCGATC**G**CG**G**CGGGGCTCACGG. The appropriate 5′ primer (100 pmol) was annealed to the RIIα cDNA with a common 3′ primer TCTCGGGGTATTGTA and *Taq* polymerase. The reaction mixture was subjected to 30 cycles of polymerase chain reaction (denaturing at 95 °C for 30 s, annealing for at 40 °C at 1.5 min, and extending at 72 °C for 2 min) to create a 644-base pair fragment. After cutting with *Nco*I and *Sal*I to excise a 90-base pair fragment encompassing the mutation each mutated insert was ligated in-frame with the remainder of the RIIα coding region in the bacterial expression plasmid pET11d (Novagen).

A mutation in the RIIβ coding region changing alanine 6 to proline (**C**CC) was produced with a 5′ primer, CGGCCATGGCC*CATATG*AGCATCGAGATC**C**CCCCGGGGCTCACGGAG, encompassing the initiation codon of rat RIIβ, a 3′ primer, GTCACCATCATCACCTTG, and *Taq* polymerase using the rat RIIβ cDNA as a template. Thirty cycles of polymerase chain reaction (denaturing at 95 °C for 30 s, annealing for at 42 °C at 1.5 min, and extending at 72 °C for 2 min) generated a 585-base pair fragment. This fragment was digested with *Nde*I and *Bam*HI to excise a 468-base pair insert encompassing the mutation and ligated into the bacterial expression vector pET 11c containing the remainder of rat RIIβ coding region.

All inserts were sequenced by the method of Sanger (18) to confirm that the correct mutations were made and that the remainder of the coding region was unaltered. Each plasmid was transfected into *Escherichia coli* BL21 DE3 cells and expression of recombinant protein was induced in the presence of 0.4 mM isopropyl-1-thio-β-thio-D-galactopyranoside for 3–4 h. Purification of recombinant RII was performed according to the method of Scott and colleagues (13) 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 ultra filtration through 10,000 nominal molecular weight limit regenerated cellulose (Amicon).

RII Overlays—RII overlays were performed according to the method of Hausken and colleagues (19) in the presence of 0.4 μM anchoring inhibitor peptide (DLIEEAAVSRIVDAVIEQVKAAGAY) or a control peptide (DLIEEAAVSR**P**VDAVIEQVKAAGAY) which is unable to block RII binding. RII competition overlays were performed according to the methods of Li and Rubin (16).

AKAP Overlays—An *in vitro* AKAP overlay was used to probe RII or mutants immobilized on nitrocellulose. Proteins (over a concentration range from 20 to 120 ng) were transferred to nitrocellulose using a Hybri-Slot manifold (BRL) and filters were blocked by incubation in a Tris-buffered saline solution (pH 7.0) containing 5% dry milk, 1% bovine serum albumin at room temperature for 1 h. Filters were incubated with ^{32}P radiolabeled AKAP-kfc fusion proteins (HT31 or MAP2). The AKAP-kfc proteins were phosphorylated by the C subunit of PKA on a consensus phosphorylation site fused to the carboxyl terminus of each protein (20). Incubation, washing, and detection procedures were identical to those described for the RII overlay (19).

AKAP peptide overlays were performed with biotinylated peptides to the RII-binding domains of Ht31 (biotin-DLIEEAAVSRIVDAVIEQVKAAGAY), AKAP95 (biotin-ETPEEVAAEVLAEVITAAVKAVEG D), or AKAP79 (biotin-YETLLIETASSLVKNALQLSIEQL). Filters were washed extensively in excess TBST buffer (three times) to remove the unbound peptides. Bound peptides were detected by incubation with streptavidin-conjugated horseradish peroxidase (0.1 μg/ml) in TBST for 1 h and visualized by chemiluminescence. Films were digitally scanned and intensities were measured using the NIH image 1.55 program.

In Situ Overlay—Human osteosarcoma MG-63 cells were grown on coverslips following the procedure as described (21). Cells were fixed with neutral formaldehyde (3.7%), permeablized with 100% methanol, and blocked with 0.5% bovine serum albumin/phosphate-buffered saline (pH 7.4) for 1 h at 4 °C. Cells were incubated with RII or mutants (80 nM) for 3 h at room temperature. Unbound RII was removed by washing in excess with phosphate-buffered saline three times (30 min).

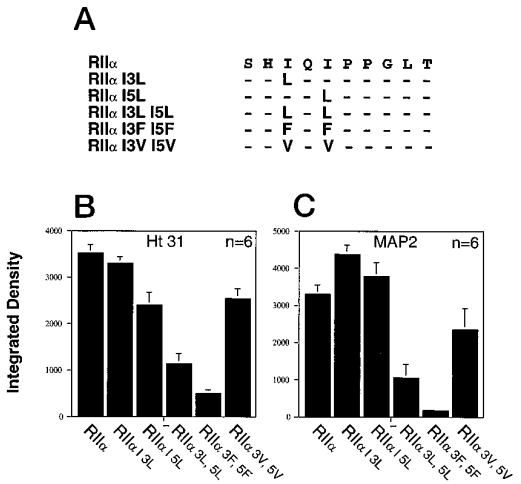

FIG. 1. **Hydrophobic side chains at positions 3 and 5 of RII are determinants for AKAP binding.** A series of five mutants were generated to probe the requirement of hydrophobic amino acids at positions 3 and 5 in each RII protomer for anchoring. *A,* amino acids are designated by the single letter code and each substitution is indicated. Aliquots of purified RIIα or mutants were immobilized onto nitrocellulose filters. Individual filters were probed with excess radiolabeled Ht31 (*B*) or MAP2 (*C*) (specific activities ranging from 2.1×10^5 to 1.5×10^5 cpm/nmol). Detection of binding was by autoradiography and was measured by densitometry of the autoradiographs. A range of RII or mutant concentrations were used to determine the linear range of binding. The degree of Ht31 (*B*) or MAP2 (*C*) binding at a single concentration RIIα or mutants (25 ng for Ht31 and 12.5 ng for MAP2) is presented. The accumulated data of six individual experiments is presented as a percent binding compared to wild-type RIIα.

Bound RII was detected immunochemically as described previously (22) using affinity purified RII antibodies (1:500). Cells were examined using a Leica confocal laser scanning system equipped with a Leitz Fluovert-FU inverted microscope and an argon/krypton laser. Confocal sections were between 1.5 and 2-μm absolute thickness. Specificity of immunostaining was confirmed by incubating control cultures with secondary antisera alone.

Co-Purification of RII·AKAP Complexes—Cell lysates (approximately 300 μg in 100 μl) from human embryonic kidney 293 cells overexpressing Ht31 were incubated with equal amounts of RIIα or RIIα I3S,I5S (5 μg). Purification of the RII·AKAP complexes was achieved by incubation with cAMP-agarose overnight at 4 °C. The affinity resin was washed in 20 column volumes of hypotonic buffer at 4 °C. RII was eluted with buffer containing 75 mM cAMP (1 ml) and co-purifying Ht31 was detected by Western blot with rabbit antiserum (1:4000).

RESULTS

Expression and Characterization of RII Mutants—RII mutants were expressed in bacteria and the properties of each recombinant protein were compared to wild-type RIIα. Each protein was purified to homogeneity by cAMP-agarose affinity chromatography, confirming that all of the mutants were able to bind cAMP. The mobility of each mutant was similar to wild-type RIIα on SDS-polyacrylamide gel electrophoresis and each protein was detected at a position which was consistent with the migration of the RII dimer on nondenaturing gels (data not shown). Previous studies have shown that monomeric or incorrectly folded RII forms migrate with a distinct mobility on nondenaturing gels (15). Other studies confirmed that each RII mutant was efficiently phosphorylated by the C subunit of PKA *in vitro* (data not shown). Collectively, these observations suggest that mutation of sites within the extreme amino-terminal regions of residues of RIIα or RIIβ have no measurable effects on certain well characterized functions of the regulatory subunits. This observation is consistent with studies showing that removal of the amino-terminal domains from RI or RII generates fragments that are functional in cAMP binding and inhibition of the C subunit (13, 23–26).

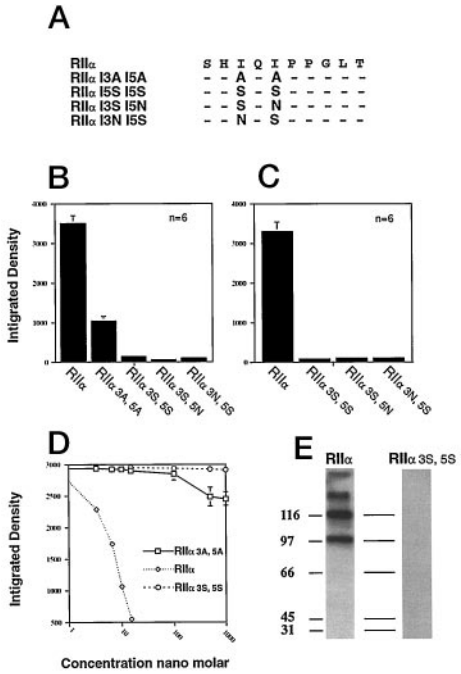

FIG. 2. **Hydrophilic side chains at positions 3 and 5 abolish AKAP binding.** A series of four RIIα mutants were generated to examine the effect of hydrophilic side chains in the AKAP-binding site. *A,* amino acids are designated by the single letter code and the position of each substitution is indicated. Aliquots of purified RIIα or mutants were immobilized onto nitrocellulose filters. Individual filters were probed with excess radiolabeled Ht31 (*B*) or MAP2 (*C*) (specific activities ranging from 2.1×10^5 to 1.5×10^5 cpm/nmol). Detection of binding was by autoradiography and was measured by densitometry of the autoradiographs. A range of RIIα or mutant concentrations were used to determine the linear range of binding. The degree of Ht31 (*B*) or MAP2 (*C*) binding at a single concentration of RIIα or mutants (25 ng for Ht31 and 12.5 ng for MAP2) is presented. The accumulated data of six individual experiments is presented as percent binding compared to wild-type RIIα. *D,* competition binding assay developed by Li and Rubin (16) was used to demonstrate that RIIα I3S,I5S was unable to compete with wild-type RIIα for binding to immobilized Ht31 (25 ng). Competition profiles over a range of concentrations (1 nM to 1 μM) of RIIα (●), RIIα I3S,I5S (—), and RIIα I3A,I5A (○) are indicated. *E,* solid-phase binding of RIIα and RIIα I3S,I5S to a variety of AKAPs expressed in MG-63 osteosarcoma cells was assessed by the standard overlay procedure (19). RII binding was detected by autoradiography.

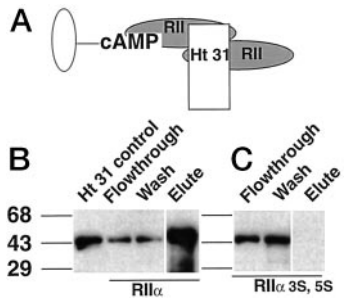

FIG. 3. **Co-purification of RII·AKAP complexes.** Equal amounts (5 μg) of RIIα or RIIα I3S,I5S were incubated with cell lysates from HEK 293 cells that overexpress a recombinant fragment of Ht31. *A,* RII or the mutant was purified by affinity chromatography on cAMP-agarose and proteins eluted with 75 mM cAMP were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide electrophoresis gel. Co-purification of Ht31 protein was detected by Western blot (41) from fractions incubated with wild-type RIIα (*B*) and RIIα I3S,I5S (*C*). The source of each sample is indicated *above* each lane.

Substitution of Hydrophobic Side Chains—While RII dimerization is a prerequisite for anchoring, we and others have demonstrated that discrete regions within the first 30 amino acids of RII participate in AKAP binding and homodimer formation (13, 15, 16). The first five residues of each RII protomer are absolutely required for anchoring and there is a 6-fold decrease in binding when isoleucines 3 and 5 are substituted to alanine (15). This suggests that AKAP-binding specificity may be conferred, in part, by the nature of the hydrophobic groups at these positions. To test this hypothesis, three RII mutants were generated where different hydrophobic side chains were introduced at positions 3 and 5 (Fig. 1*A*). Substitution of isoleucines with valine investigated whether a β-branched hydrophobic group was necessary for AKAP-binding, whereas substitution with leucine or phenylalanine tested the functionality of long-chain or bulky hydrophobic groups (Fig. 1*A*). Changes in AKAP binding were measured by a semi-quantitative overlay procedure which we have previously used to monitor changes in the anchoring affinities of RII mutants (15, 19).

Introduction of leucine at position 3 decreased Ht31 binding by 6 ± 4% ($n = 6$) when compared to wild-type RIIα (Fig. 1*B*). Substitution at position 5 decreased binding by 32 ± 8% ($n = 6$). Surprisingly, single substitution of either leucine slightly enhanced MAP2 binding (Fig. 1*C*). However, binding to either Ht31 or MAP2 was markedly reduced when both isoleucines were replaced with leucine. Ht31 binding was decreased by 74 ± 5% ($n = 6$) and MAP2 binding was decreased by 69 ± 8% ($n = 6$) (Fig. 1, *B* and *C*). These results suggest β-branched groups at positions 3 and 5 are critical for AKAP binding. Further support for this hypothesis was provided by the analysis of RII mutants where phenylalanine was introduced at both positions. The intent of these experiments was to test the tolerance of bulky hydrophobic groups at both positions. RIIα I3F,I5F bound both AKAPs to a lesser extent than the double leucine mutant (Fig. 1, *B* and *C*). Ht31 binding was decreased 86 ± 2% ($n = 6$), whereas a 94 ± 2% ($n = 6$) decrease in MAP2 binding was recorded (Fig. 1*C*). Presumably, the diminished MAP2 binding affinity of RIIα I3F,I5F reflects subtle topological differences in the RII-binding sites on MAP2 and Ht31. This provides indirect evidence for the notion that there may be slight differences in the RII binding preferences of individual AKAPs (27, 28). However, this effect may be enhanced by the introduction of four bulky hydrophobic side chains per RII dimer which combine to sterically hinder vital contacts between the regulatory subunit and MAP2.

In order to test whether the length of a β-branched side chain was important for anchoring we measured the AKAP-binding affinity of RIIα mutants with valines substituted at positions 3 and 5. Introduction of valine minimally decreased Ht31 binding affinity by 28 ± 6% ($n = 6$) and MAP2 binding by 29 ± 12% ($n = 6$) (Fig. 1, *B* and *C*). Thus, the β-branched character of the isoleucine and valine side chains rather than the length of the aliphatic chain seems to confer an advantage in AKAP binding. It is likely that these branched side chains interact with a complimentary hydrophobic face on the amphipathic helix of the anchoring protein. This view is supported by mutagenesis studies showing that hydrophobic side chains in the amphipathic helix region of AKAP75 participate in RII binding (16).

Hydrophilic Substitutions at Positions 3 and 5 Abolish AKAP Binding—To establish whether side chain character was a contributing factor in anchoring, neutral or hydrophilic side chains were introduced at positions 3 and 5 in each RII protomer (Fig. 2*A*). These RII mutants exhibited a dramatic decrease in AKAP binding (Fig. 2, *B* and *C*). For example, substitution of serine at both positions reduced Ht31 binding by 96 ± 1% ($n = 6$) and reduced MAP2 binding by 98 ± 1% ($n = 6$) as assessed by a semi-quantitative RII overlay (Fig. 2, *B* and *C*). Introduction of asparagine at either position gave similar results (Fig. 2, *B* and *C*). To confirm these measurements by a

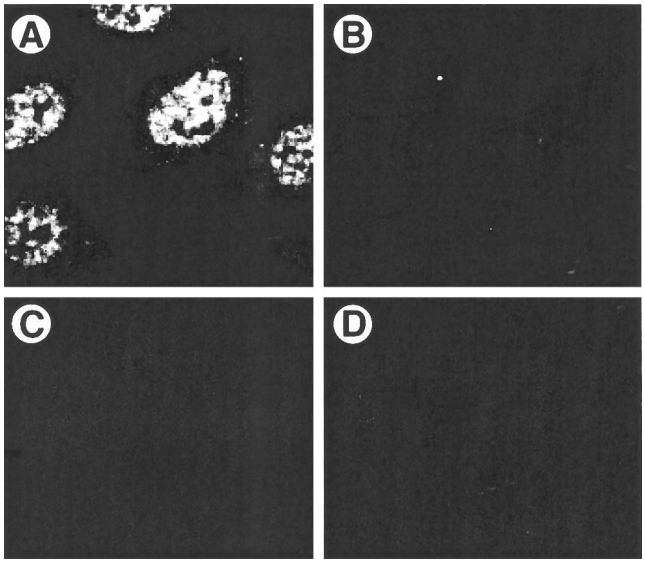

FIG. 4. **Fluorescent detection of RII binding *in situ*.** Human MG-63 osteosarcoma cells were seeded on coverslips and grown in culture for 36 h. After fixation with 3.7% formaldehyde and permeablization with 100% (w/v) methanol, unoccupied RII-binding sites were detected by an *in situ* overlay procedure that as described under "Materials and Methods." Detection of anchored murine RII was achieved by indirect immunofluorescence using fluorescein-conjugated rabbit anti-goat secondary antibodies (1:100 dilution). Cells were incubated with the same concentration (80 nM) of wild-type murine RIIα8 (*A*), murine RIIα I3S,I5S (*B*), murine RIIα + 1 μM anchoring inhibitor peptide (*C*), and in the presence of affinity purified anti-murine RII antisera alone (*D*) (1:2,000 dilution).

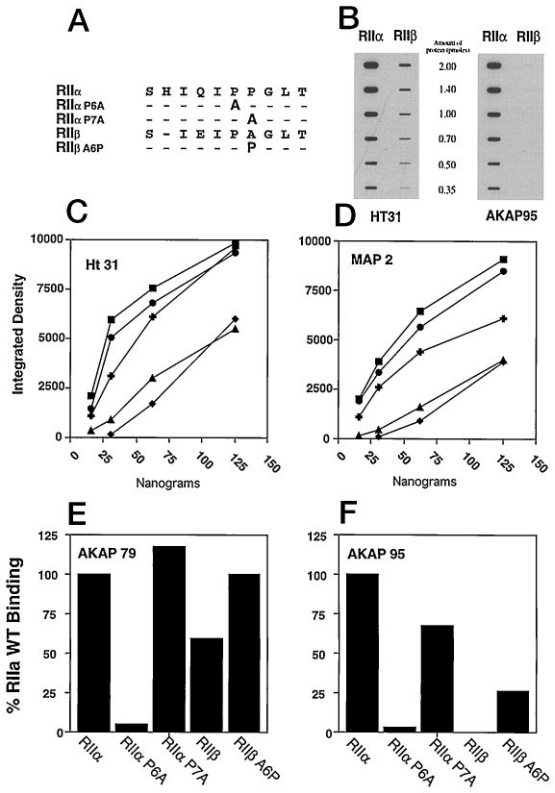

FIG. 5. **Isoform differences and determinants for AKAP binding.** The first 10 residues of RIIα and RIIβ are aligned. *A*, amino acids are indicated in the single letter code. A family of RII mutants were generated to examine the role of prolines as determinants for isoform selective association with AKAPs. *B,* the binding properties of wild-type RIIα and RIIβ (over a range of concentrations from 2 to 0.35 pmol) were compared by a solid-phase AKAP overlay using biotinylated Ht31 peptide or AKAP95 peptides (0.4 μM) as probes. Biotinylated peptide was detected by chemiluminescence using streptavidin-coupled horseradish peroxidase. Using similar approaches the binding properties of both RII isoforms and three mutants were measured using radiolabeled Ht31 (*C*) or radiolabeled MAP2 (*D*), biotinylated AKAP79 peptide (*E*), and biotinylated AKAP 95 peptide (*F*) as probes. *Panels C* and *D* are representative experiments from a series of 8 individual measurements that depict binding properties of radiolabeled AKAP proteins (specific activities ranging from 2.1×10^5 to 1.5×10^5 cpm/nmol) over a range of concentrations from 15 to 125 ng. Detection of RIIα (■), RIIβ (▲), RIIα P6A (♦), RIIα P7A (●), and RIIβ A6P (+) was by autoradiography and quantitation was achieved by densitometry using the NIH 1.5 scan plus program. *Panels E* and *F* are representative experiments from four individual measurements that depict the binding properties of both RII isoforms and mutants at a single concentration (80 ng) to AKAP79 peptide (*E*) and AKAP95 peptide (*F*). Detection of bound peptide was as described above. The results are presented as the percentage of wild-type RIIα binding.

second independent method, we used an RII competition assay (16). Various concentrations of RIIα or mutants (1 nM to 1 μM) were incubated with radiolabeled RIIα (5 nM) and solid-phase Ht31 binding was measured by standard techniques. RIIα I3A,I5A began to compete with wild-type RIIα for Ht31 binding at higher concentrations (Fig. 2*D*). However, RIIα I3S,I5S was unable to compete with wild-type RIIα even at a 200-fold molar excess of mutant (Fig. 2*D*). Further analysis demonstrated that ^{32}P radiolabeled RIIα I3S,I5S was unable to bind AKAPs expressed in MG-63 osteosarcoma cells as assessed by the RII overlay, whereas the wild-type protein detected proteins ranging in size from 97 to 220 kDa (Fig. 2*E*). In summary, these experiments show that RIIα I3S,I5S is unable to bind AKAPs as assessed by several variations of the solid-phase overlay assay (19, 29).

Additional experiments were conducted to determine whether RIIα I3S,I5S could interact with AKAPs in solution. RIIα I3S,I5S or wild-type RIIα were mixed with cell extracts from HEK 293 cells expressing Ht31 and complexes were isolated by affinity chromatography on cAMP-agarose (Fig. 3*A*). Ht31 co-purified with wild-type RII (Fig. 3*B*) but not with the RIIα I3S,I5S mutant (Fig. 3*C*). Similar results were obtained when co-purification experiments were performed with another anchoring protein AKAP79 (data not shown). In order to examine the properties of RIIα I3S,I5S in a more cellular context, we used an *in situ* technique where RII or the mutant was used to detect anchoring sites in cells (30, 31). *In situ* binding of wild-type RIIα to sites in the nucleus, cytoplasmic, and perinuclear regions was detected in MG-63 human osteosarcoma cells (Fig. 4*A*). In contrast, very little staining was detected in cells incubated with the same concentration of RIIα I3S,I5S (Fig. 4*C*). All RII binding was blocked when overlays were performed in the presence of excess (0.4 μM) anchoring inhibitor peptide (Fig. 4*D*). There is no background staining in the experiments because endogenous human RII is not detected by the antibody (Fig. 4*B*). Collectively, these studies show that RIIα I3S,I5S is a non-localizable RII mutant.

RII Isoform Selective AKAP Binding—Several years ago, Liesner and colleagues (10) suggested that both RII isoforms, RIIα and RIIβ, had different AKAP binding preferences. These investigators concluded that RIIα had a 6-fold preference for MAP2 whereas RIIβ had a 2-fold preference for AKAP79 (10). This finding could have important physiological implications for PKA anchoring as it suggests a mechanism for differential localization of type II PKA isoforms within cells. Sequence comparison of RIIα and RIIβ show that there is some divergence within the first 10 amino acids of both proteins (Fig. 5*A*). On the basis of this and our previous studies with RIIα we proposed that isoform selective AKAP binding determinants should be located in the extreme NH_2-terminal regions of each RII protomer. A testable aspect of this theory is that RIIα and

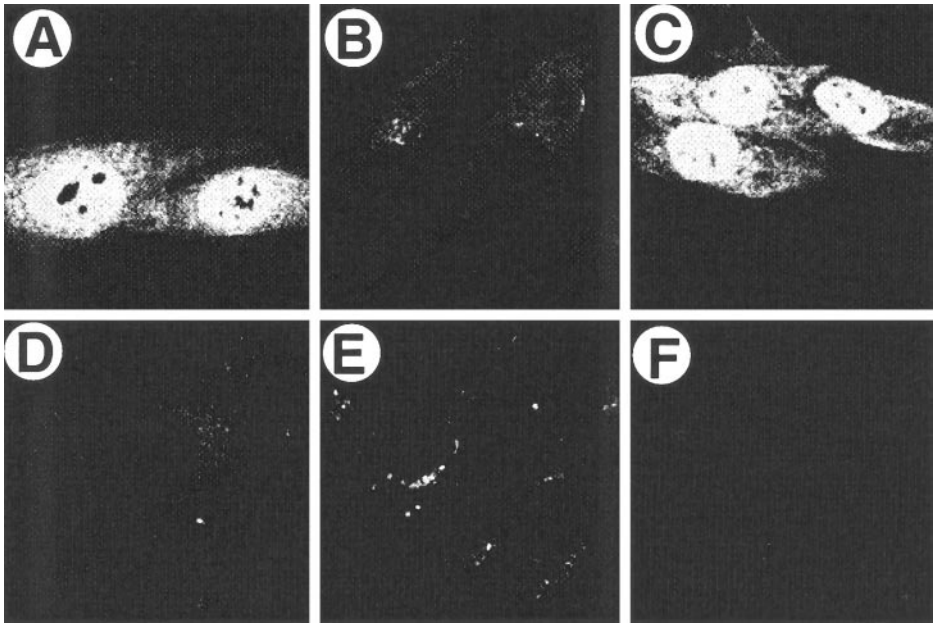

FIG. 6. **Mutation of proline 6 impairs RII anchoring *in situ*.** Human MG-63 osteosarcoma cells were seeded on coverslips and grown in culture for 36 h. Cells were treated under the same conditions as described in the legend of Fig. 5. Cells were incubated with RII or mutants at a concentration of 80 nM murine RIIα (*A*), RIIα P6A (*B*), RIIα P7A (*C*), RIIβ (*D*), RIIβ A6P (*E*), and RIIα + 0.4 μM anchoring inhibitor peptide (*F*). Detection of anchored murine RII was achieved by indirect immunofluorescence using fluorescein-conjugated rabbit anti-goat secondary antibodies (1:100 dilution).

RIIβ should have measurable differences in their binding affinities for individual AKAPs. Therefore, we decided to use a reverse overlay procedure to screen the RII-binding regions of two AKAPs for isoform selective RII binding preferences (19). Peptides from Ht31 and AKAP95 bound RIIα (2 to 0.35 pmol) with equal affinity, whereas RIIβ was unable to bind the AKAP95 peptide even at the highest concentrations used in the assay (Fig. 5*B*).

Two side chains in the first 10 amino acids of RIIα are not conserved in RIIβ (Fig. 5*A*). Glutamine at position 4 was considered unlikely to represent an isoform selective anchoring determinant because mutations at this position to alanine have no effect on AKAP binding for either RII isoform (15, 16). However, RIIα contains a proline pair at positions 6 and 7 that is not present in RIIβ. To test the role of these prolines two RIIα mutants were generated: RIIα P6A where proline 6 was substituted with alanine and RIIα P7A where proline 7 was substituted for alanine. RIIα P6A exhibited reduced binding for all of the AKAPs tested (Ht31, MAP2, AKAP79, and AKAP95) as assessed by the semi-quantitative overlay assay (Fig. 5, *C-F*). Decreased AKAP binding was measured in a range from 57 $\pm$ 8% (n = 8) for Ht31 to 97% (n = 4) for AKAP95. This was supported by band shift analysis confirming that RIIα P6A was unable to shift the mobility of an Ht31 fragment even at a 4-fold higher concentration than wild-type RIIα (data not shown). In contrast, substitution of proline 7 had only a modest effect on binding to any of the AKAPs tested (Fig. 5, *C-F*).

In order to test whether the prolyl pair could enhance AKAP binding affinity, reciprocal experiments were performed where an additional proline was introduced at the corresponding position in RIIβ. This mutant, RIIβ A6P, exhibited increased Ht31 binding affinity by a factor of 1.4 $\pm$ 0.1-fold (n = 8) relative to wild-type RIIβ (Fig. 5*C*). Comparable increases in binding to MAP2 and AKAP79 were also observed (Fig. 5, *D* and *E*). Moreover, the RIIβ A6P mutation conferred limited AKAP95 binding which was 26% (n = 4) of that observed for RIIα (Fig. 5*F*).

The AKAP binding properties of these RII isoform mutants were further tested using the *in situ* overlay. Significant nuclear, cytoplasmic, and perinuclear staining was detected in cells probed with wild-type RIIα and RIIα P7A (Fig. 6, *A* and *C*). However, staining was excluded from the nucleus and decreased in cytoplasmic and perinuclear regions when RIIα P6A or RIIβ were used as probes (Fig. 6, *B* and *D*). These results are consistent with the decreased AKAP binding observed for RIIα P6A and RIIβ relative to RIIα *in vitro*. Our finding that RIIβ A6P binds AKAPs with higher affinity than RIIβ wild-type is also supported by the *in situ* overlay technique which detects increased perinuclear staining with RIIβ A6P as probe (Fig. 6*E*). In contrast, little, if any nuclear staining was detected for RIIβ A6P despite the binding to AKAP95 detected by peptide overlay. This discrepancy could be accounted for by differences in the two experimental techniques combined with the 4-fold lower AKAP95 binding detected for this mutant relative to RIIα wild-type. Nevertheless, the results of both *in vitro* and *in situ* binding studies suggest that the prolines at positions 6 and 7 play a crucial role in determining the AKAP binding specificity for RIIα.

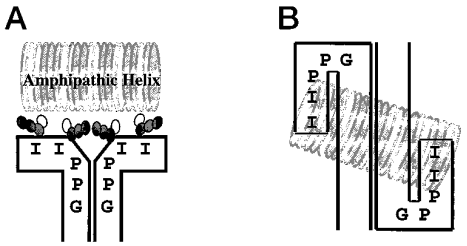

FIG. 7. **Models for the RII-AKAP interaction.** A schematic diagram depicting two possible topologies of the RII-AKAP interaction. *A,* parallel orientation of the RII dimer-AKAP interaction domain. Isoleucine side chains are depicted by *circles* with the *open circles* representing the β-branch methyl group. *B*, anti-parallel orientation of the RII dimer-AKAP interaction domain.

DISCUSSION

The mutagenesis and binding studies presented in this report expand our original observation that AKAP binding determinants are located in the extreme amino-terminal regions of each RII protomer (13, 15). More specifically we have extended our original observations in three ways: β-branched side chains at positions 3 and 5 are positive determinants for AKAP binding, the introduction of hydrophilic side chains such as serine or asparigine completely abolish anchoring, and a proline-proline pair at positions 6 and 7 increase or stabilize RIIα interaction with selected anchoring proteins. Our first postulate is supported by evidence that removal of isoleucine and replace-

ment with its non-branched isomer, leucine, significantly reduced anchoring. This observation is compatible with studies by Glanz *et al.* (32) who have shown that leucines and isoleucines on AKAP75 are required for RII binding. Hence, it is possible that the isoleucine side chains on RII interlock with their reciprocal partners on a hydrophobic face of the AKAP. This type of docking may be similar to the hydrophobic interactions that maintain the leucine zipper in transcription factors such as C/EBP and CREB (33, 34). However, it should be noted that the protein-protein interactions required for RII-AKAP interactions are more complicated and involve three polypeptide chains: the amphipathic helix on the AKAP and both RII protomers. Other investigators have proposed that downstream sites provide additional contact with the anchoring protein (16). While this seems a likely conclusion, the extent and contribution of these additional sites for high affinity RII-AKAP interaction is unclear as the RIIα 13S,I5S mutant has no measurable AKAP binding affinity.

Our second postulate is that non-localizable mutants, such as RIIα I3S,I5S, may turn out to be valuable reagents to elucidate AKAP function by acting as dominant-negative effectors of PKA anchoring when expressed in mammalian cells. The RIIα I3S,I5S protein is likely to be suited for this function as it seems to retain the wild-type functions such as responsiveness to cAMP and inhibition of the C subunit. Furthermore, all indications suggest that RII I3S,I5S is a stably folded protein implying that this mutant RII form will have a normal half-life in cells. Potentially, a non-localizable RII subunit will be a more versatile reagent than anchoring inhibitor peptides which are currently being used to compete with AKAPs to displace the type II PKA from anchoring sites (35, 36). The predominant limitation of these peptides is their poor membrane permeability and questionable stability inside cells. Another advantage of RIIα I3S,I5S as a probe to investigate the physiological significance of PKA anchoring may be to overexpress the non-localizable mutant in cells which lack R subunits. This type of venture is now possible as McKnight and colleagues (37–40) have created mouse strains where individual PKA genes have been knocked out. The availability of cell lines derived from these mice will make it possible to examine the physiological roles of individual R subunits and the effects of an anchoring mutant such as RIIα I3S,I5S on selected cAMP responsive events.

Our third postulate proposes that prolines at positions 6 and 7 adapt RIIα for preferential association with certain AKAPs. Specifically, our results suggest that replacement of proline 6 with alanine (RIIα P6A) decreases the preferential binding of RIIα for four anchoring proteins. The most dramatic reduction in binding was measured for the nuclear matrix associated anchoring protein AKAP95 (31). This decrease in binding to AKAP95 is supported by the *in situ* overlay studies demonstrating that RIIα P6A is unable to occupy nuclear binding sites in MG-63 cells. Overall, RIIα P6A exhibits AKAP binding properties more similar to those observed for RIIβ including staining of exclusively perinuclear regions in MG-63 cells. Correspondingly, introduction of a proline pair at the equivalent position in RIIβ results in a mutant protein (RIIβ A6P) which exhibits an AKAP binding spectrum similar to RIIα including acquired binding to AKAP95.

Surprisingly, our results show that AKAP79 binds RIIα with a 2-fold preference over RIIβ. However, a previous study detected a 2-fold preference of the bovine homolog AKAP75 for RIIβ (10). These differences may be attributed to trace amounts of RIIα in the brain preparations used for the previous study or differences in the detection methods used in either study. Nevertheless, we propose that the structural characteristics imposed by tandem prolines at positions 6 and 7 play a role in determining the AKAP binding specificity for RIIα. The occurrence of the proline pair may allow RIIα to be the preferred binding partner for certain anchoring proteins such as AKAP95 or a 80-kDa anchoring protein that is induced in granulosa cells upon exposure to follicle-stimulating hormone (40). Although a precise structural explanation for these observations is not available, it seems plausible that a proline at position 6 may increase RIIα affinity for certain AKAPs in one of two ways. Either through direct contact of proline 6 with the anchoring proteins, or through the added rigidity of the imino peptide linkage between isoleucine 5 and proline 6 functioning to precisely orient AKAP binding determinants such as isoleucines 3 and 5. Some support for this later explanation is provided by molecular modeling of the NH_2-terminal region of RII which suggests proline 6 may occupy a specific position within a β-turn.

Despite the role that the prolines may play in positioning anchoring determinants, it is clear that the orientation of the RII dimer is also an essential component of the AKAP-binding site as it controls the spatial organization of isoleucines 3 and 5 (15). Unfortunately, none of the studies to date have been able to distinguish between a parallel or antiparallel orientation for the RII dimer (13–16). A parallel dimer would cluster the essential isoleucines on each RII protomer to form a hydrophobic face that contacts the AKAP, while the rigidity of an imino linkage provided by each proline may function to segregate the anchoring determinants at positions 3 and 5 from the downstream residues required for homodimer formation (Fig. 7*A*). In contrast, an antiparallel orientation would place a pair of isoleucines at either end of the AKAP-binding site. In this context, prolines may participate in a β-turn that orients the isoleucine pair on each RII protomer (Fig. 7*B*). If the antiparallel model is correct the additional proline present in RIIα would lock each RII protomer into a conformation where the upstream anchoring determinants are folded back on the remainder of the dimerization surface. This conformation could increase the surface area available for contact with the AKAP. Undoubtedly, the completion of structural studies to solve the solution structure of an RII fragment complexed with the Ht31 peptide should define the orientation of the RII dimer and the precise topology of the of AKAP-binding surfaces on RII.

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