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Association of the Type II cAMP-dependent Protein Kinase with a Human Thyroid RII-anchoring Protein

CLONING AND CHARACTERIZATION OF THE RII-BINDING DOMAIN*

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The type II cAMP-dependent protein kinase (PKA) is localized to specific subcellular environments through binding of the dimeric regulatory subunit (RII) to anchoring proteins. Subcellular localization is likely to influence which substrates are most accessible to the catalytic subunit upon activation. We have previously shown that the RII-binding domains of four anchoring proteins contain sequences which exhibit a high probability of amphipathic helix formation (Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. E., Brennan, R. G., and Scott J. D. (1991) *J. Biol. Chem.* 266, 14188–14192). In the present study we describe the cloning of a cDNA which encodes a 1015-amino acid segment of Ht 31. A synthetic peptide (Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Gln-Val-Lys-Ala-Ala-Tyr) representing residues 493–515 encompasses the minimum region of Ht 31 required for RII binding and blocks anchoring protein interaction with RII as detected by band-shift analysis. Structural analysis by circular dichroism suggests that this peptide can adopt an α-helical conformation. Both Ht 31 (493–515) peptide and its parent protein bind RIIα or the type II PKA holoenzyme with high affinity. Equilibrium dialysis was used to calculate dissociation constants of 4.0 and 3.8 nM for Ht 31 peptide interaction with RIIα and the type II PKA, respectively. A survey of nine different bovine tissues was conducted to identify RII binding proteins. Several bands were detected in each tissues using a ^{32}P-RII overlay method. Addition of 0.4 μM Ht 31 (493–515) peptide to the reaction mixture blocked all RII binding. These data suggest that all anchoring proteins bind RIIα at the same site as the Ht 31 peptide. The nanomolar affinity constant and the different patterns of RII-anchoring proteins in each tissue suggest that the type IIα PKA holoenzyme may be specifically targeted to different locations in each type of cell.

Since its discovery by Sutherland and colleagues (1), considerable research has focused on elucidating the action of cAMP as an intracellular messenger for hormone-mediated events. Many hormones, prostaglandins, and neurotransmitters act through parallel pathways that elevate intracellular cAMP and lead to activation of the cAMP-dependent protein kinase (PKA)[1] (2). An apparent paradox exists in that parallel pathways can all activate the same enzyme, PKA, although it is clear that individual hormones can trigger distinct and diverse physiological processes in the same cell (3, 4). In essence, each hormone elevates cAMP concentrations but promotes PKA-mediated phosphorylation of distinct substrate proteins (5, 6). One hypothesis to explain these observations is that individual hormones activate specific pools of PKA activity (2). A potential mechanism to explain this phenomenon is that individual PKA pools are compartmentalized at their site of action close to their preferred substrates and become active only when the appropriate hormone elevates cAMP in that particular microenvironment (7, 8). For this to occur, PKA must be maintained at the correct intracellular loci in close proximity to its preferred substrates. In recent years it has become evident that PKA is compartmentalized at specific sites throughout the cell via the interaction of its regulatory subunit (R) with specific anchoring proteins (9–14).

Although RII-anchoring proteins may play a central role in cAMP-mediated signal transduction, little is known about their structure, subcellular location, or tissue distribution. So far, only two classes of RII-anchoring proteins have been characterized in any detail. Cytoskeletal attachment of type II PKA occurs through interactions between RII and microtubule-associated protein 2 (MAP2) (15, 16). The site on MAP2 that contacts RII has been identified as a 31-residue peptide in the amino-terminal region of the molecule (17, 18). RII also associates with a bovine brain calmodulin-binding protein designated P75 (11). Several P75 analogs, ranging in size from M_r 60,000 to 150,000, have been reported in different species and may represent members of a family of structurally related RII-anchoring proteins (11, 19). The partial cDNA clones for two members of this family, P150 and P75, have been isolated from bovine and murine brain libraries, respectively (19, 20). These molecules contain a conserved carboxyl-terminal domain of 117 amino acids but have distinct amino terminal sequences (20, 21). Sequences at the extreme carboxyl terminus of these proteins are required for anchoring since deletion mutants lacking carboxyl-terminal 15 amino

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M90360.

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; MAP2, microtubule-associated protein 2; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

acids of P75 or 26 amino acids of P150 are unable to bind RIIβ (19, 20). Comparison of the last 30 residues of P75 or P150 with the 31 amino acid RII-binding region of MAP2 revealed no conserved primary structure. However, computer generated searches for similar secondary structures identified regions of 14 amino acids in each molecule which display high probabilities of amphipathic helix formation (22).

Previous studies have shown that dimerization of RII is required for anchoring protein interaction (23), and that only the first 50 amino acids on RII are required for binding (23, 24). It is likely, therefore, that the anchoring protein interacts with both RII protomers (22). Accordingly, the topology of an anchored type II PKA holoenzyme complex could place both catalytic subunits in close proximity to the anchoring-protein, allowing its rapid and preferential phosphorylation following catalytic subunit (C) activation. Based upon this model, we predict that most RII-anchoring proteins will be substrates for PKA and contain a conserved amphipathic helix in the RII-binding domain. This report documents the cloning and characterization of a cDNA for Ht 31. This cDNA encodes a 1015-amino acid fragment which contains multiple phosphorylation sites for several of the multifunctional protein kinases, including PKA. The RII-binding domain of Ht 31 has been incorporated into a 24-residue peptide which binds RIIα or the type II PKA holoenzyme with nanomolar affinity. This peptide can adopt an α-helical conformation as assessed by circular dichroism and is a potent competitor of PKA anchoring as it can block all solid-phase RIIα/binding protein interaction.

EXPERIMENTAL PROCEDURES

Purification of Proteins—Recombinant RIIα was purified from *Escherichia coli* and phosphorylated by incubation with bovine catalytic subunit and [^{32}P]ATP as we have previously described (23).

Screening of λgt11 Libraries—A human thyroid cDNA library of 5 × 10^5 independent clones was screened using the technique of Bregman *et al.* (19). Nitrocellulose filters from 10 plates (50,000 plaques/plate) were prepared for antibody screening by the method of Helfman *et al.* (25). Filters were probed with ^{32}P-RIIα to detect high affinity RII-anchoring proteins using the overlay procedure of Lohman and colleagues (13) with modifications documented by Bregman *et al.* (19).

Sequencing of Clones—cDNA inserts from positive colonies were isolated and subcloned into the *Eco*RI site of Bluescript KS vector for restriction mapping and sequencing. Nucleotide sequencing was performed by the dideoxy chain-termination method of Sanger *et al.* (26).

Construction and Expression of Ht 31 Fragments—Bluescript plasmid containing a cDNA insert (3.1 kilobase pairs) encoding Ht 31 (BS Ht 31), was digested with *Nco*I and *Bam*HI releasing a 941-base pair fragment which was isolated and subcloned into the expression vector pETkfc. The *E. coli* expression vector pETkfc is an adaptation of the T7 polymerase system reported by Studier *et al.* (27). KFC is a DNA cassette encoding a 51-residue peptide containing a consensus PKA phosphorylation site, a Factor X cleavage site, and a calmodulin-binding peptide which was engineered into the *Bam*HI site of the bacterial expression vector pET11d[2] (Novagen). Expression of proteins using the pETkfc vector system was as described by Studier and colleagues (27) and produced fusion proteins with a carboxyl-terminal calmodulin binding-affinity tail. Each fusion protein was purified by affinity chromatography on calmodulin-Sepharose by the method of Sharma and colleagues (28). Purified proteins were concentrated by ultrafiltration through Amicon Centricon microconcentrators.

Site-directed Mutagenesis—All mutagenesis was performed using the "altered sites™" *in vitro* mutagenesis system (Promega Biotec). The full-length Ht 31 cDNA was subcloned into the *Bam*HI site of the phagemid vector pSELECT-™. This vector contains the origin of replication, and single-stranded DNA can be recovered after infection of cultures with the M13K07 helper phage. Single-stranded pSELECT templates were obtained by infection of JM109 liquid cultures growing in mid-log phase, and, after overnight growth at 37 °C, the bacteriophage were harvested and DNA was isolated. Double-primer mutagenesis was performed with the mutagenic oligonucleotide (21 mers) as described in the "altered sites™" manual. All mutations were confirmed by nucleotide sequencing of the entire coding region. Mutated DNA was cut from the appropriate pSELECT vector by digestion with 5 units of *Nco*I and *Bam*HI and ligated into a similarly digested pETkfc bacterial expression vector. Each mutant protein was expressed and purified using the pETkfc vector system as described above.

Design of Mutants—Mutants to disrupt the secondary structure of regions flanking putative amphipathic helix regions in Ht 31 were generated by substituting proline residues for amino acids upstream or downstream of the helix. Single-point mutations in Ht 31 were introduced at Lys-490 (AAG), Leu-494 (TTG), Ala-512 (GCC), and Leu-517 (CTG) using mutagenic oligonucleotides of 21 bases. The underlined nucleotides indicate those changed to produce the proline codon of (CC*X*).

Peptide Synthesis, Purification, and Iodination—Peptides were synthesized at the University of Oregon peptide synthesis facility. Cleavage from the resin and deprotection was performed by Multiple Peptide Systems, San Diego. The peptide was purified by reverse-phase HPLC using a SynChrom C-18 column (250 × 10 mm, inner diameter) on a Beckman HPLC system. The solvent system was trifluoroacetic acid/acetonitrile. The concentration and composition of the peptide was confirmed by amino acid analysis using an Applied Biosystems Analyzer. Ht 31 (493–515) peptide was synthesized with a tyrosine as the carboxyl-terminal residue, allowing the incorporation of ^{125}I by the chloramine-T method (29).

RII Overlay Procedure—Solid phase binding overlays were performed by the method of Lohman and colleagues (13) with modifications documented by Bregman *et al.* (19).

Band-shift Analysis—The ability of Ht 31 mutants to form complexes with RIIα or the type II PKA holoenzyme was assessed by nondenaturing gel electrophoresis. The RIIα-Ht 31 complex migrates slower than either free RII or Ht 31 and could be easily distinguished from the individual components. Polyacrylamide gels were prepared as described by Laemmli (30) with the omission of SDS in all reagents. Binding reactions were conducted in buffer containing 5 mM HEPES (pH = 7.2), 10 mM dithiothreitol, 1 mM benzamidine HCl, 100 mM KCl, and 0.01% Tween 20. Samples were incubated for 1 h at 22 °C (23 μl total volume), diluted with 100 mM MOPS (pH 6.8), 40% glycerol, and 0.014% bromphenol blue (7 μl) and loaded on the gel. Free and complexed proteins were separated by electrophoresis on 6% (w/v) polyacrylamide gels at 40 mA for 3 h at 4 °C. Protein bands were detected using Fast Stain™ (Zoion Research) or by autoradiography.

Circular Dichroism Analysis—Circular dichroism spectra were recorded using a Jasko J/500A spectropolarimeter for solutions containing 50 μM Ht 31 (493–515) peptide in 35 mM potassium phosphate buffer (pH 7.0), and 50% trifluoroethanol. Spectra from 180 to 260 nm were analyzed for secondary structure by computer comparison with spectra of proteins of known structure.

Equilibrium Dialysis—The dissociation constants (K_d) between RII and Ht 31 (493–515) peptide was determined by equilibrium dialysis. Radiolabeled Ht 31 (493–515) peptide was produced to a specific activity of 5 μCi/mmol. Dialysis was performed in a Spectrum equilibrium dialyzer equipped with microcells using Spectra/Por 2 12–14,000 MWCO membranes. Equilibrium is reached by rotating the cell overnight at room temperature. The cell volume was 400 μl, 200 μl of reaction mixture are added to each side of the membrane. The binding reaction buffers contain 5 mM HEPES (pH = 7.2), 10 mM dithiothreitol, 1 mM benzamidine HCl, 100 mM KCl, and 0.01% Tween 20, the same as used for gel shift analysis. After equilibrium is reached, 20-μl samples from each side of the membrane are extracted through ports in the cells and counted in a Packard gamma counter. The total amount of free ligand was calculated after correcting for the number of moles of ligand bound to the membrane and cell walls. The amount of ligand bound to RII was calculated as described by Klotz (31) and the affinity constants were determined using a Scatchard plot (32).

RESULTS

Cloning of Ht 31 cDNA—Since RII-anchoring proteins bind to common or overlapping sites within the first 50 amino

[2] Stofko-Hahn, R. E., Carr, D. W., and Scott, J. D. (1992) *FEBS Lett.*, in press.

acids of the regulatory subunit dimer (23, 24), we proceeded to identify the RII-binding domains in several molecules and compare their sequences for a region of common structure. Using the modified RII overlay procedure of Bregman and colleagues (19), a human thyroid λgt11 expression library (provided by Dr. Roger Cone, Vollum Institute) was screened for RII-binding proteins. Six positive clones were identified from 5×10^5 plaques. Each positive clone was subcloned into the *Eco*RI restriction site of Bluescript KS vectors for sequence analysis (as described in under "Experimental Procedures"). One cDNA, designated Ht 31, was sequenced on both strands using unique synthetic oligonucleotide primers. The sequence contains a continuous open reading frame which encodes a protein fragment of 1015 amino acids. The nucleotide sequence (3045 base pairs) and predicted primary structure of Ht 31 are presented in Fig. 1. Comparison of the Ht 31 sequence with the GenBank™ and Protein Identification Resource sequence databases did not identify any overall homology with other proteins. However, residues 494–507 of Ht 31 are 43% identical to sequences found within the RII-binding domains of three other RII-anchoring proteins MAP2, P150, and Ht 21. We have previously reported that these regions have a high probability to form amphipathic helices (22).

Binding of Ht 31 and MAP2 to RIIα and the Type II PKA Holoenzyme—Our model for subcellular localization of PKA predicts that the type II holoenzyme (R2C2) must interact with RII-anchoring proteins with similar affinity as the RII dimer alone. Therefore, it was important to demonstrate that Ht 31 binds to both RIIα and the type II PKA holoenzyme. Recombinant RIIα was incubated with an excess of bovine C subunit to allow formation of the type II PKA holoenzyme. Holoenzyme formation was detected by a shift in migration rate of protein bands on a nondenaturing polyacrylamide gel.

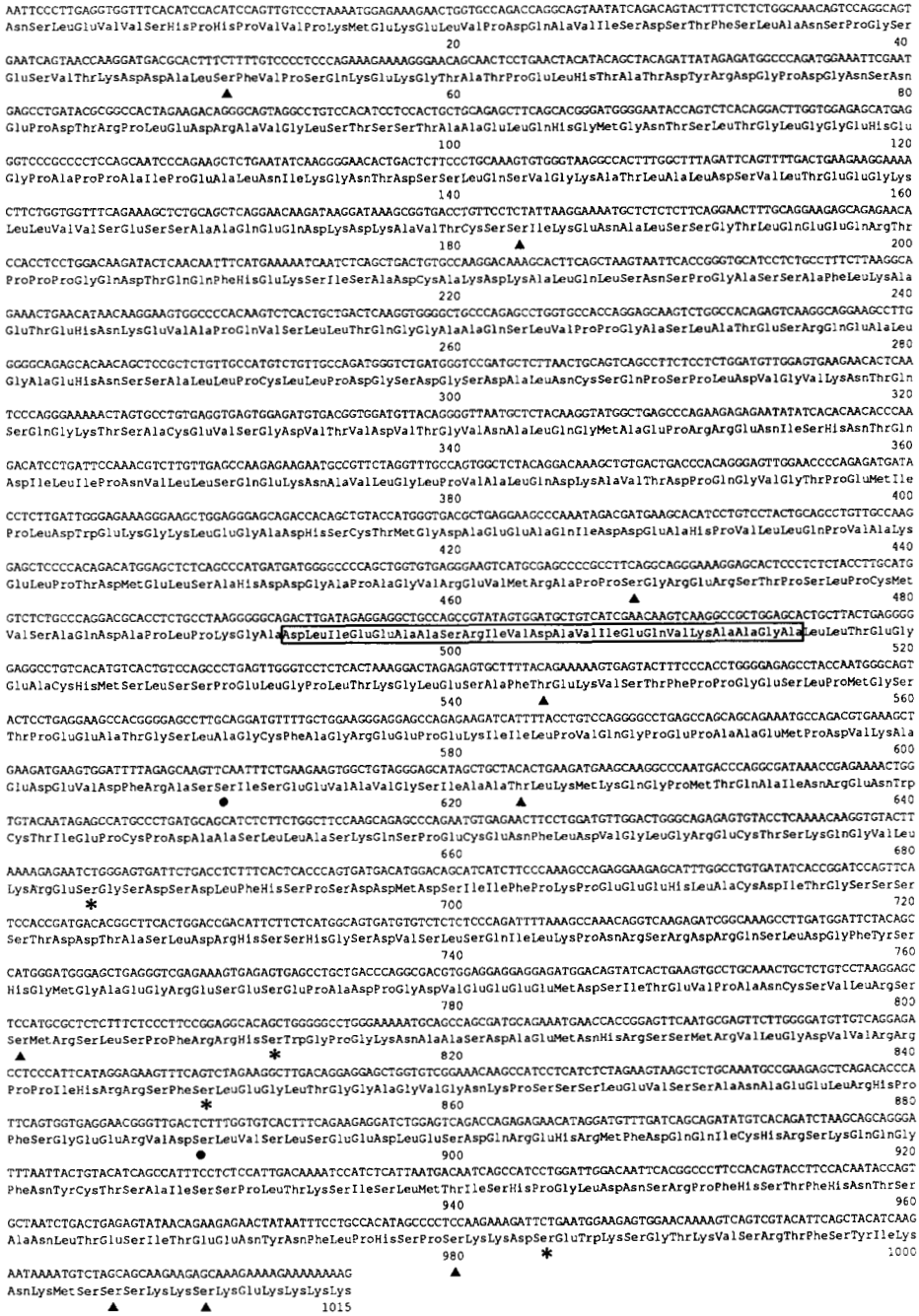

FIG. 1. **Sequence characterization of the Ht 31 cDNA.** The figure shows the nucleotide sequence (*upper line*) and deduced amino acid sequence (*lower line*) of a cDNA encoding the human thyroid RII-anchoring protein Ht 31. The *boxed region* indicates the RII-binding domain. Consensus substrate sites for cAMP-dependent protein kinase (*asterisks*), protein kinase C (*triangles*), and Ca^{2+}/calmodulin kinase II (*circles*) are indicated *below* the target amino acids.

The catalytic subunit is basic (pI = 8.3) and runs at the top of the gel, while the regulatory subunit is acidic (pI = 4.6) and migrates much faster (Fig. 2, *lanes 1* and *2*). When R and C are incubated together before running on the gel, a new band appears between the C and R bands (Fig. 2, *lane 3*). The formation of the holoenzyme complex can be prevented by addition of 100 μM cAMP (data not shown). A recombinant fragment of Ht 31, Ht 31 (418–736), was incubated with C, RIIα, or the type II PKA holoenzyme. Incubation of Ht 31 with RIIα produces a protein complex which migrates to a distinct position (Fig. 2, *lane 6*). Similarly, the Ht 31-PKA holoenzyme complex migrates with a slower mobility (Fig. 2, *lane 7*). The presence of the C subunit alone does not alter the mobility of Ht 31 (Fig. 2, *lane 4* and *5*). Similar results were obtained when a recombinant fragment of human MAP2 (1–154) was incubated with RIIα or the type II PKA holoenzyme (Fig. 2, *lanes 8–11*). As a control, Ht 31 Pro-507, a mutant protein unable to bind RII, was used in these experiments and did not complex with either RIIα or the type II PKA holoenzyme (data not shown). These results show that RII-binding fragments of two anchoring proteins, Ht 31 (418–736) and MAP2 (1–154), bind equally well to either the regulatory subunit or the type II PKA holoenzyme.

Analysis of the RII-binding Domain in Ht 31—Residues 494–507 of Ht 31 predict an amphipathic helix which is a determinant for RII binding (22). In order to define the minimum sequence capable of high affinity interaction with RIIα, point mutations flanking this region were produced in the Ht 31 (418–736) fragment. Selected residues were mutated to proline and the mutant fragments were expressed in the kfc vector system. Mutation of either lysine 490 or leucine 494 to proline (Fig. 3*B*, *lanes 2* and *3*) had no significant effect upon RII binding as assessed by an RII-overlay procedure (Fig. 3*B*). Mutation of alanine 512 to proline significantly reduced RII binding (Fig. 3*B*, *lane 4*), suggesting that residues immediately carboxyl-terminal of the putative amphipathic helix region may include additional binding determinants. As a control leucine 517 was mutated to proline (Fig. 3*B*, *lane 5*) and had no measurable effect upon RII/Ht 31 interaction. Since mutations at lysine 490, leucine 494, or leucine 517 did not effect RII binding, it suggests that site-directed substitutions of proline into this region did not destabilize the overall conformation of the Ht 31 protein. In summary, the segment of sequence between residues 494 and 516 of Ht 31 appears to be the minimum structure necessary for interaction with RII. To confirm this conclusion, a peptide corresponding to this region of sequence Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Gln-Val-Lys-Ala-Ala-Gly-Ala-Tyr was synthesized. Aspartic acid 493 was included to enhance the solubility of the synthetic RII-binding peptide and a tyrosine at the carboxyl terminus was added to permit radiolabeling with ^{125}I.

Binding of Ht31 (493–515) Peptide to RII—Direct binding of ^{125}I-labeled Ht 31 (493–515) peptide to RIIα was detected by band-shift analysis (Fig. 4, *A* and *B*). The peptide alone runs with the dye front and cannot be detected by Coomassie staining (Fig. 4*A*). However, if ^{125}I-labeled peptide is preincubated with RIIα, the RII-peptide complex can be detected by autoradiography (Fig. 4*B*). Binding of the peptide does not significantly alter the migration rate of RII (compare *lanes 1* and *2*, Fig. 4*A*). Ht31 (418–736) fragment, which does produce a shift in migration when complexed to RII (Fig. 4*A*, *lanes 3–*

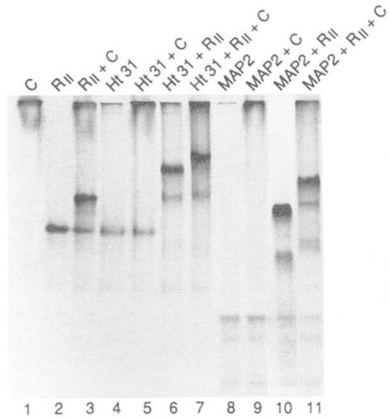

FIG. 2. **Binding of recombinant RII-anchoring protein fragments to RIIα and type II PKA holoenzyme under nondenaturing conditions.** The ability of Ht 31 (418–736) and MAP2 (1–154) to interact with RIIα or the type II PKA holoenzyme was determined by band shift assay. The migration rate of the proteins or protein complexes is based on size and charge. Complex formation produces a band with a migration rate distinct from any of the components. Various combinations of proteins (5 μg each) were mixed together for 1 h at 22 °C before electrophoresing on a 6% (w/v) polyacrylamide gel and staining with Coomassie Brilliant Blue. *Lane 1* contains C subunit (the C subunit is quite basic and migrates to the top of the lane); *lane 2*, RIIα; *lane 3*, RIIα + C (type II PKA holoenzyme); *lane 4*, Ht 31; *lane 5*, Ht 31 + C; *lane 6*, Ht 31 + RIIα; *lane 7*, Ht 31 + type II PKA holoenzyme; *lane 8*, MAP2; *lane 9*, MAP2 + C; *lane 10*, MAP2 + RIIα; *lane 11*, MAP2 + type II PKA holoenzyme.

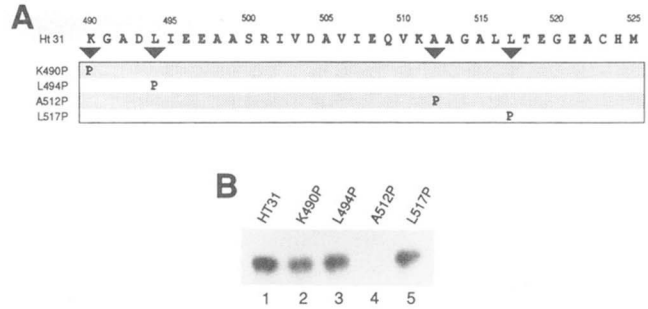

FIG. 3. **Analysis of the RIIα-binding domain on Ht 31.** *A*, amino acids within the RII-binding domain of Ht 31 are shown with *arrows* indicating residues mutated to proline. Point mutations were produced in the Ht 31 cDNA and expressed in plasmid pETkfc. Mutant Ht 31 proteins were partially purified from bacterial extracts and separated by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and electrotransfered to immobilon membrane. The blot was analysed by autoradiography (*B*) after incubation with ^{32}P-RIIα. *Lane 1* contains Ht 31 wild type, and mutants K490P, L494P, A512P, and L517P are in *lanes 2–5*, respectively.

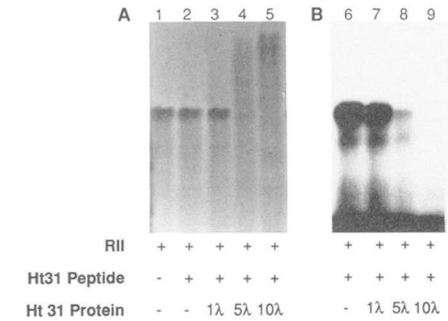

FIG. 4. **Band-shift analysis of RIIα/Ht 31 peptide interaction and competition with Ht 31 protein fragment.** ^{125}I-Labeled Ht 31 (493–515) peptide (4.2 ng/μl) and RIIα (4.2 μg/μl) were incubated for 15 min at 22 °C followed by addition of the indicated amounts of Ht 31 protein (0.5 μg/μl). Samples were then incubated for an additional 15 min before loading on a 6% (w/v) nondenaturing polyacrylamide gel. Gels were analyzed by Coomassie Blue staining (*A*) and autoradiography (*B*). The reaction mixtures are indicated *below* each *lane*.

5), displaces the peptide from RII in a dose-dependent manner (Fig. 4*B*, *lanes 6–9*). A control peptide, patterned after a calmodulin binding amphipathic helix in the Ca^{2+} calmodulin kinase II (provided by Dr. Tom Soderling, Vollum Institute) did not bind to RIIα (data not shown).

Effect of Peptide on Interaction of RII with Various Bovine Tissue-binding Proteins—Nine bovine tissues were analyzed by overlay assay for RIIα-binding proteins (Fig. 5*A*). Several RII-binding bands were detected in each tissue and some tissues, such as lung, brain and testis, contain many RII anchoring proteins. Addition of Ht 31 (493–515) peptide (0.4 μM) to the overlay buffer completely blocked RIIα interaction with all anchoring proteins (Fig. 5*B*). The same concentration of Ht 31 peptide blocks RII interaction with purified protein fragments of MAP2 and another recombinant thyroid RII-anchoring protein, Ht 21 (data not shown). In contrast, the addition of a control amphipathic helix peptide, mastoporan (0.4 μM), to overlay buffers had no effect on RII binding. These results suggest that Ht 31 (493–515) peptide binds RII at the same or overlapping sites as other RII-anchoring proteins.

Circular Dichroism Analysis of Ht 31 (493–515) Peptide—Computer generated prediction of secondary structure predicted that Ht 31 (493–515) peptide could adopt an α-helical conformation. When the peptide was depicted using a helical-wheel projection (not shown) the hydrophilic and hydrophobic amino acids were aligned on opposite faces. This suggested that the peptide may form an amphipathic helix. Therefore, the conformation of Ht 31 (493–515) peptide was analyzed by circular dichroism. In a solution containing 50% trifluoroethanol the peptide had a significant amount, 55%, of α-helical content (Fig. 6). Even in the absence of trifluoroethanol the peptide maintained a conformation that included 17% α-helical content (data not shown). These data are consistent with this region of the Ht 31 protein adopting an α-helical conformation.

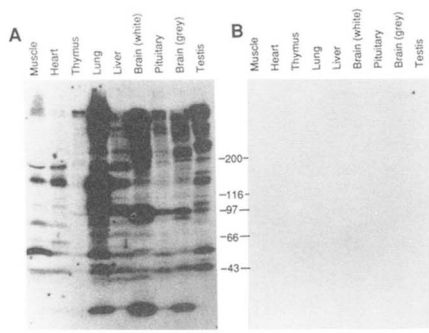

FIG. 5. **Tissue survey of bovine RII-anchoring proteins.** Crude protein extracts from nine bovine tissues were analyzed by RII overlay for anchoring proteins. Protein samples (50 μg) were separated by electrophoresis on a 4–12% SDS-polyacrylamide gradient gel. After electrotransfer to immobilon, RII-binding proteins were detected as described under "Experimental Procedures." Two identical blots were incubated with either ^{32}P-RIIα (*A*) or ^{32}P-RIIα in the presence of 0.4 μM Ht 31 (493–515) peptide (*B*). Tissue sources are marked *above* each *lane*.

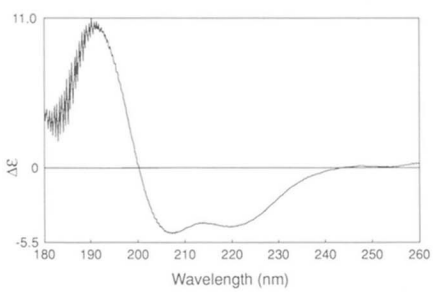

FIG. 6. **Circular dichroism spectra of Ht 31 (493–515) peptide.** Circular dichroism spectrum of Ht 31 (493–515) peptide was measured as described under "Experimental Procedures."

Calculation of the Dissociation Constant (K_d) of Ht 31 (493–515) Peptide for RIIα and the Type II PKA—The binding affinities of ^{125}I-Ht 31 (493–515) peptide for RIIα and type II PKA holoenzyme were determined by equilibrium dialysis. The dissociation constants (K_d) for RIIα/Ht 31 (493–515) peptide and type II PKA holoenzyme/Ht 31 (493–515) peptide interaction were calculated by Scatchard analysis as 4.0 nM ± 1.2 (n = 6) and 3.8 nM ± 1.8 (n = 2), respectively (Fig. 7, *A* and *B*). Addition of cAMP (100 μM) to the reaction buffer does not significantly change the binding constant (data not shown). These data suggest that Ht 31 (493–515) peptide has a high affinity for RIIα and that the presence of the C subunit or cAMP does not affect binding affinity.

DISCUSSION

The cAMP-dependent protein kinase is a multifunctional enzyme which is activated in response to numerous hormones and neurotransmitters. PKA-mediated phosphorylation controls many diverse cellular processes and therefore, the kinase must be highly regulated to ensure that individual hormones bring about phosphorylation of the appropriate target protein. Subcellular localization of the type II PKA is one possible mechanism to allow the kinase to perform specific functions in a variety of tissues by determining which substrates are most accessible to the catalytic subunit upon activation. The cloning and characterization of Ht 31 has provided valuable reagents permitting a detailed and quantitative analysis of RII/anchoring protein interaction. Evidence presented in this report suggests that a considerable proportion of the type IIα PKA may be specifically localized through high affinity interaction with anchoring proteins. First, a 24-residue peptide derived from Ht 31, the human thyroid RII-anchoring protein, binds RIIα with nanomolar affinity. Second, a survey of nine bovine tissues identified numerous RII-anchoring proteins in each tissue (Fig. 5*A*).

Binding studies using a synthetic peptide have confirmed that the essential determinants for high affinity interaction with RIIα lie within a linear sequence of 23 residues located between residues 493 and 515 of Ht 31. Circular dichroism analysis of Ht 31 (493–515) peptide suggests that the spatial arrangement of determinants for RII binding are maintained by an α-helical conformation (Fig. 6). Circular dichroism data support, but do not prove, our previous prediction that residues 493–507 of Ht 31 can form an amphipathic helix (22). We are currently working on co-crystallizing the Ht31 peptide with RII to provide definitive proof of the secondary structure of the RII-binding domain on Ht 31. If the RII-binding sequence does exist in an α-helical conformation, four acidic side chains Asp-493, Glu-497, Glu-498, and Asp-504 are aligned on one face of the helix while hydrophobic residues are oriented to the other face. Side chains located between residues 508 and 513, which lie outside the predicted helix region, enhance RII binding and may function to stabilize the overall conformation of the region.

The majority of previous studies with RII-anchoring proteins have concentrated on interaction with RII alone and have not considered the influence that the C subunit may have on type II PKA holoenzyme binding (15–23). Moreover, a model we have recently proposed for subcellular localization of PKA predicts that the type II holoenzyme (R2C2) must interact with RII-anchoring proteins with similar affinity as the RII dimer alone. In support of this model, it was critical

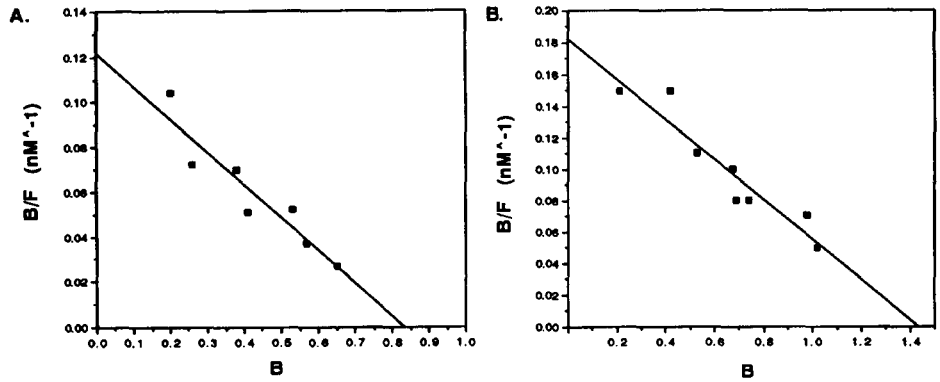

FIG. 7. **Scatchard analysis of the binding of Ht 31 (493–515) peptide to RIIα or the type II PKA holoenzyme.** Data for Scatchard analysis of the binding of Ht 31 (493–515) peptide to RIIα (*A*) or the type II PKA holoenzyme (*B*) were measured by equilibrium dialysis as described under "Experimental Procedures." The data are graphed as B/F (moles of peptide bound per mol of RII/concentration of free peptide (nM)) *versus* B (moles of peptide bound per mol of RII).

to demonstrate that Ht 31 bound with high affinity to both RIIα and the type II PKA holoenzyme. Using a band-shift technique, we have shown that Ht 31 and MAP2 protein can complex with the type II PKA holoenzyme (Fig. 2*A*, *lanes 7* and *11*). While this technique demonstrates qualitatively that the two different RII-anchoring proteins can bind the type II holoenzyme, it provides no information about the affinity of interaction. Therefore, an important extension of this work was to measure the affinity of Ht 31 for RIIα and the type II holoenzyme. These experiments were not technically feasible with Ht 31 protein, but Ht 31 (493–515) peptide was an ideal ligand for these studies because of its low molecular weight and high solubility. Equilibrium dialysis was used to determine binding constants (K_d) of 4.0 and 3.8 nM for the interaction of Ht 31 (493–515) peptide with RIIα and the type II PKA holoenzyme respectively (Fig. 7). These values are not statistically different and show that the C subunit does not significantly influence binding of RII to the anchoring protein. Furthermore, the equilibrium dialysis data imply that Ht 31 protein can bind PKA with high affinity and that both proteins will be co-localized *in vivo*.

Northern blot analysis, using Ht 31 cDNA as a probe, has identified a mRNA species of 7 kilobase pairs.[3] This confirms that the cDNA we have isolated (3045 base pairs) is a partial clone representing approximately half of the full length of the molecule. The nucleotide sequence of Ht 31, presented in Fig. 1, contains a single open reading frame extending throughout the entire cDNA which encodes a central portion of the protein fragment of calculated M_r 108,222. This sequence of 1015 amino acids does not have any significant identity with other known proteins in the PIR data base, nor does it contain any defined sequence motifs such as leucine zippers, transmembrane domains, or nuclear localization signals which could indicate the possible subcellular location of Ht 31. Work is currently underway to obtain cDNA clones which encode the remainder of the Ht 31 molecule whose sequence may provide information about the function or subcellular location of Ht 31. Future studies are planned to determine the tissue distribution and expression level of Ht 31 using antibodies that are currently being raised against a recombinant fragment of the protein.

Ht 31 contains numerous potential phosphorylation sites for the multifunctional protein kinases PKA, PKC, and Ca^{2+}/calmodulin-dependent kinase II (33, 34). The majority of these sites are grouped in the carboxyl-terminal portion of the molecule and carboxyl-terminal of the RII-binding domain (Fig. 1). There are four consensus PKA phosphorylation sites (Lys/Arg-Lys/Arg-Xaa-Ser-Xaa) located at positions 684, 812, 849, and 984 in the sequence (Fig. 1). So far we have shown that a recombinant fragment of Ht 31 which contains only one of these putative phosphorylation sites, serine 684, is phosphorylated by PKA *in vitro*.[4] Potentially, PKA phosphorylation at any of these sites may unmask secondary activities residing in the Ht 31 protein and may be a mechanism to trigger an individual cAMP-mediated response. Because of their close association with the kinase, RII-anchoring proteins can undergo rapid and preferential phosphorylation in response to cAMP and may be important substrates. This concept is supported by evidence that most RII-anchoring proteins are PKA substrates (13). MAP2 is phosphorylated by PKA at multiple sites, incorporating up to 11 molecules of phosphate (15) while P150 and P75 are *in vitro* substrates for the kinase (11). We have cloned another RII-anchoring protein, AKAP 79, which is phosphorylated by PKA *in vitro* and contains a cluster of three consensus PKA phosphorylation sites within a 20-residue region of sequence (21).

Previous reports have suggested that both RII isoforms, RIIα and RIIβ, may have different subcellular locations (35). The RIIβ isoform is selectively expressed in the brain and neuroendocrine tissues and may adapt the type IIβ kinase for regulation of metabolism and cell functions in the central nervous system (20). Up to 65% of the type IIβ kinase is associated through interaction with anchoring proteins to particulate components of the cerebral cortex, cytoskeletal components and organelles (14, 36, 37). RIIα has also been reported to be associated with the particulate fraction, with the ratio of soluble/particulate kinase varied from one tissue to another and one species to another (12, 14, 36). The evidence presented in this and other reports (11, 22) suggests that subcellular anchoring also adapts the type IIα kinase for regulation of specific cellular functions. Binding of Ht 31 to the type IIα holoenzyme occurs with high affinity (K_d = 3.8 nM) and the tissue distribution of RII-anchoring proteins is not restricted to neural tissues. More than 40 RIIα-binding bands, ranging in size from M_r 25,000 to 300,000 were detected by overlay experiments (Fig. 5*A*). Although some of these bands presumably represent proteolyzed RII-binding fragments derived from larger precursors, the number and tissue distribution of RII-anchoring proteins is still considerably greater than we originally anticipated. The majority of these anchoring proteins must associate with RIIα since the expression of RIIβ is restricted to the central nervous system (14). If PKA localization is a physiologically relevant mechanism for targeting specific substrates, disruption of RII/anchoring protein interaction should have a specific effect on selected cAMP responsive events. As is graphically demonstrated in Fig. 4*B*, Ht 31 (493–515) peptide is a potent competitor of PKA anchoring. An important aspect of these experiments is that all bands detected by the RII-overlay procedure are prevented from interaction with RIIα by the addition of 0.4

[3] T. Jahnsen, personal communication.

[4] I. D. C. Fraser and J. D. Scott, unpublished observation.

μM Ht 31 (493–515) peptide. This is compelling evidence to support the notion that all anchoring proteins bind to the same or overlapping sites on RII. Based on these studies, it should be feasible to use this peptide to disrupt the normal localization of PKA in a variety of tissues by microinjection of peptide or expression of a mini-gene encoding the RII-binding site.

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