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Interaction of the Regulatory Subunit (RII) of cAMP-dependent Protein Kinase with RII-anchoring Proteins Occurs through an Amphipathic Helix Binding Motif*

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Daniel W. Carr‡, Renata E. Stofko-Hahn‡, Iain D. C. Fraser‡, Sarah M. Bishop‡, Ted S. Acott§¶, Richard G. Brennan¶, and John D. Scott‡¶‖

From the ‡Vollum Institute for Advanced Biomedical Research and the Departments of §Ophthalmology and ¶Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, Oregon 97201-3098

The type II cAMP-dependent protein kinase is localized to specific subcellular environments through the binding of the regulatory subunit (RII) dimer to RII-anchoring proteins. Computer-aided analysis of secondary structure, performed on four RII-anchoring protein sequences (the microtubule-associated protein 2, P150, and two thyroid proteins Ht 21 and Ht 31), has identified common regions of approximately 14 residues which display high probabilities of forming amphipathic helices. The potential amphipathic helix region of Ht 31 (Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile) lies between residues 494 and 507. A bacterially expressed 318-amino acid fragment, Ht 31 (418–736), containing the amphipathic helix region, was able to bind RIIα. Site-directed mutagenesis designed to disrupt the secondary structure in the putative binding helix reduced binding dramatically. Specifically, substitution of proline for Ala-498 significantly diminished RIIα binding, and similar mutation of Ile-502 or Ile-507 abolished interaction. Mutation of Ala-522 to proline, which is located outside the predicted amphipathic helix region, had no effect on RIIα binding. These data suggest that anchoring proteins interact with RIIα via an amphipathic helix binding motif.

Cyclic AMP serves as the second messenger for numerous hormones and neurotransmitters (1). The cAMP-signaling pathway is composed of individual hormone receptors and adenylate cyclases which are coupled physically through interaction with intermediary GTP-binding proteins (2). Hormone binding to its receptor stimulates adenylate cyclase, resulting in the production of cAMP (2–4). Four molecules of cAMP can bind each dormant PKA[1]-holoenzyme complex (R_2C_2), thereby causing release of two active catalytic (C) subunits from the regulatory (R) subunit dimer (4, 5). Although many hormones utilize the cAMP-signaling pathway to activate PKA, individual hormones produce diverse biochemical effects (4). A potential mechanism to explain this phenomenon is that individual hormones promote activation of PKA pools, which are localized to specific subcellular sites (6).

Subcellular localization of PKA has been shown to be directed through the R subunit (7). Two R subunit classes exist, RI and RII, which form the type I and type II holoenzymes, respectively (8, 9). Type II PKA is present in all cells, whereas the tissue distribution of type I PKA is more restricted (10, 11). The subcellular distributions of both PKA subtypes are also distinct. The RI isoforms (RIα and RIβ) are primarily cytoplasmic, while certain tissues contain up to 75% of either RII isoform (RIIα or RIIβ) in particulate form, associated with either the plasma membrane, cytoskeletal components, secretory granules, or the nuclear membranes (9, 12–18). Type II PKA localization is dictated by the association of RII with specific anchoring proteins (12–15). Tissue-specific patterns of RII-anchoring proteins have been detected by protein-blotting techniques or by fractionation on RII-Sepharose affinity columns (19, 20). Presumably, tissue-specific PKA function is determined by RII-anchoring proteins which tether the holoenzyme to particular subcellular sites. This localization of PKA determines which substrates are most accessible to the C subunit upon activation by cAMP (6).

Cytoskeletal attachment of PKA occurs through interactions between RII and microtubule-associated protein 2 (MAP 2) (21). Recent reports have shown that the interaction between RII and MAP 2 occurs through the first 79 amino acids of RII (22, 23). Furthermore, binding to MAP 2 requires sites on both protomers of the RII dimer (22). In complementary studies, the site on MAP 2 that binds RIIβ has been localized to a 31-residue peptide (24, 25). The RII-binding domain in P150, a murine brain RII-anchoring protein, has been identified but not defined in detail and is believed to involve the last 15 amino acids of the protein (19). Although there is no obvious sequence similarity between MAP 2 and P150, it is quite possible that both molecules contain common secondary structural motifs which permit interaction with RII. Amphipathic helices (an α-helix with opposing polar and nonpolar faces) are known to be involved in both intra- and intermolecular protein-protein interactions (26). We have used helical wheel analysis to identify potential amphipathic helices in MAP 2, P150, and two RII-anchoring proteins (Ht 21 and Ht 31) we have recently characterized. In this paper, we demonstrate that Ht 31 and truncated proteins of Ht 31, containing

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‖ To whom correspondence should be addressed: Vollum Institute for Advanced Biomedical Research, L474, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97201-3098. Tel.: 503-494-4652; Fax: 503-494-6972.

[1] The abbreviations used are: PKA, type II cAMP-dependent protein kinase; MAP, microtubule-associated protein; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

the amphipathic helix region, bind to RIIα. Furthermore, disruption of the putative helix regions in Ht 31, by mutation of selected residues to proline, diminishes or abolishes RIIα binding activity. Therefore, we propose that RII-anchoring proteins contain amphipathic helices that are necessary for binding to the regulatory subunit of PKA and thereby dictate this kinase's subcellular location.

EXPERIMENTAL PROCEDURES

Purification of Proteins—Recombinant RIIα was purified from *Escherichia coli* as previously described (22). Fragments of the human thyroid RIIα-anchoring protein Ht 31 and related point mutants were expressed in the *E. coli* expression vector pETkfc[2] which is an adaptation of the T7 polymerase system reported by Studier *et al.* (27). A DNA cassette encoding a 51-residue peptide containing a concensus PKA phosphorylation site, Factor X cleavage site, and a calmodulin-binding peptide 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.

Construction and Expression of Ht 31 Fragments—A 941-base pair *Nco*I-*Bam*HI fragment encoding Ht 31 was subcloned into the expression vector, pETkfc. The recombinant plasmid, pETHt31kfc, was propagated in an *E. coli* host cell line, DH5α. For protein expression, the recombinant plasmid was transformed into the lysogenic strain of *E. coli*, BL21(DE3)pLysS, and 1-liter cultures were grown as described by Studier and colleagues (27).

Site-directed Mutagenesis—All mutagenesis was performed using the "altered sites™" *in vitro* mutagenesis system (Promega). The full-length Ht 31 cDNA was subcloned into the *Bam*HI site of the phagemid vector pSELECT™. Single-stranded pSELECT templates were obtained after infection of JM109 liquid cultures in midlog phase with R408 helper phage. After overnight growth at 37 °C, the bacteriophage were harvested and DNA was isolated. Double primer mutagenesis was performed with the mutagenic oligonucleotides (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 *Nco*I and *Bam*HI and ligated to a similarly cut pETkfc bacterial expression vector. Each mutant protein was expressed and purified using the pETkfc vector system as described above.

Design of Mutants—Mutants were designed to disrupt the putative α-helix conformation in Ht 31 by substituting proline residues for hydrophobic amino acids in this region. Single point mutations in Ht 31 were introduced at Ala-498 (GCT), Ile-502 (ATA), Ile-507 (ATC), and Ala-522 (GCC) using mutagenic oligonucleotides of 21 bases. Double mutations at Ala-498 and Ile-507 were introduced by simultaneously using two mutagenic oligonucleotides in the mutagenesis reactions. A triple mutation of Ht 31, introducing prolines at positions 498, 502, and 507, was performed using the double mutant Ht 31 Pro-498/502 as the template and changing Ile-507 to proline.

RII Overlay Procedure—The RII overlay binding assay was done according to the method of Lohmann and colleagues (20) incorporating those modifications documented by Bergmann *et al.* (19).

Gel-shift Analysis—The ability of Ht 31 mutants to complex with RIIα was assessed by nondenaturing polyacrylamide gel electrophoresis. The migration rate of the RIIα-Ht 31 complex is slower than either free RIIα or Ht 31 and can be easily distinguished on the gel. Polyacrylamide gels were prepared as described by Laemmli (29) 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 30 min at 20 °C (23 μl total volume), diluted with 7 μl of 100 mM MOPS (pH 6.8), 40% glycerol, and 0.014% bromphenol blue, and immediately 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 10 °C. Protein bands were detected using Fast Stain™ (Zoion Research).

[2] R. E. Stofko, D. W. Carr, and J. D. Scott, manuscript in preparation.

RESULTS

Identification of Putative Amphipathic Helices in Ht 31, Ht 21, P150, and MAP 2—By using the modified RII overlay procedure of Bergmann and colleagues (19) a human thyroid λgt11 expression library was screened for RII-anchoring proteins. Two cDNA clones encoding RII-anchoring proteins were isolated and designated Ht 21 and Ht 31.[3] The nucleotide sequence of Ht 21 contains a continuous open reading frame of 1,281 base pairs which encodes a protein of 427 amino acids. The COOH-terminal 107 amino acids are 79% identical to the murine RII-anchoring protein P150 (19). Since conserved structure often reflects conserved function, it seemed likely that this region contained the RII-anchoring domain. Furthermore, previous studies suggest that the last 15 amino acids of P150 are required for RII anchoring (19). The RII-anchoring site of MAP 2 has been localized to a region of 31 residues in the amino terminus of the molecule (24, 25). Comparison of the Ht 21 and P150 sequences with the RII-anchoring site of MAP 2 revealed no striking sequence homology. However, as has been observed for other classes of functionally similar proteins, *e.g.* calmodulin-binding proteins, it is possible that the RII-binding site in each protein is conserved in its secondary structure.

Computer-aided secondary structure predictions for Ht 31, Ht 21, P150, and MAP 2, performed by the methods of Eisenberg and colleagues (30), were used to identify a region in each protein which displays a high probability for amphipathic helix formation. These regions are up to 14 residues in length and consist of alternating pairs of hydrophobic and hydrophilic amino acids (Fig. 1*A*). Sequence comparison of the four regions revealed little homology, with only one amino acid, a glutamic acid at position 3, conserved in all the sequences (Fig. 1*A*). However, comparison of polar and nonpolar residues in this region reveals a striking alignment of hydrophilic and hydrophobic residue types (Fig. 1*B*). When each sequence was drawn as a helical wheel with 3.6 residues/turn, the hydrophilic and hydrophobic residues became grouped on opposite faces (Fig. 1*B*). No other sequences in these proteins could be similarly aligned, suggesting these are functionally relevant regions in each molecule.

Identification of Putative Amphipathic Helices and the Minimum Region of Ht 31 Required for RII Binding—The analysis of the protein sequence of Ht 31 (1035 amino acids) for potential amphipathic helices identified a region between residues 494 and 507 (Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile) (Fig. 1*A*). This region of Ht 31 is 43% identical in sequence to the binding domain of the RII-anchoring protein MAP 2 (Fig. 1*A*) and is also 43% identical to Ht 21/P150, although different amino acids are identical. To determine whether residues 494–507 of Ht 31 are involved in RII binding, a 319-amino acid fragment of Ht 31, representing residues 418–736 of the protein, was expressed in *E. coli*. Ht 31 (418–736) bound RIIα as assessed by solid-phase binding (Fig. 2). Furthermore, Ht 31 (486–717), a 232-residue Ht 31 fragment which contained the amphipathic region at the extreme amino terminus, also bound RII (data not shown). These results are compatible with our hypothesis that the amphipathic helix region of Ht 31 is necessary for RII binding.

Disruption of RII-Ht 31 Interaction by Mutagenesis within the Amphipathic Helix Region—To determine if an intact α-helix is required for RII binding, a family of Ht 31 point mutants was produced in the Ht 31 (418–736) fragment (Fig. 3*A*). The introduction of proline, especially toward the COOH terminus of an α-helix conformation, often disrupts the sec-

[3] I. Fraser, R. E. Stofko, D. W. Carr, and J. D. Scott, manuscript in preparation.

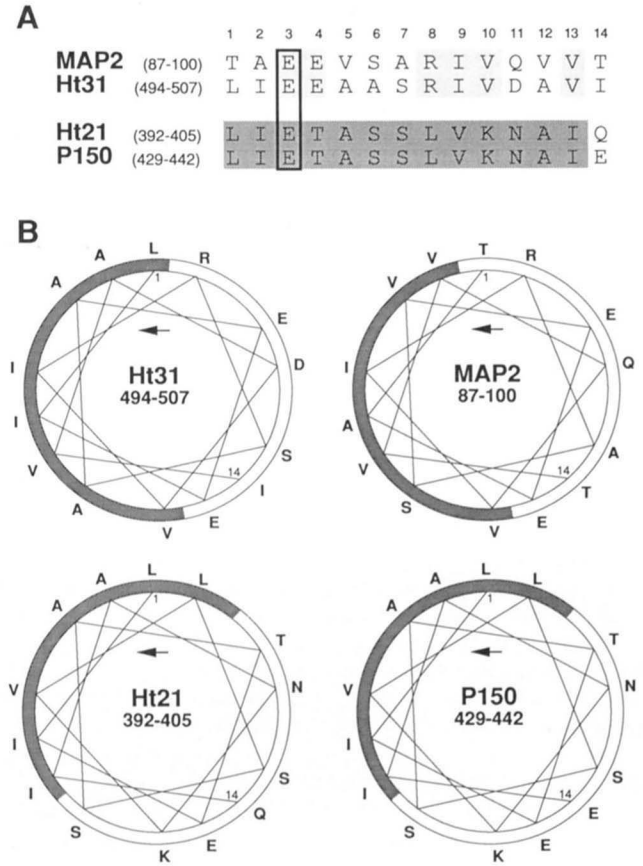

FIG. 1. **Sequence comparison and helical wheel analysis of RII-anchoring proteins.** *A*, the sequences of four RII-anchoring proteins, MAP 2 (residues 87–100), Ht 31 (residues 494–507), Ht 21 (residues 392–405), and P150 (residues 429–442) are aligned. The invariant glutamic acid residue present in all four proteins is *boxed*, *light shaded areas* indicate homology between MAP 2 and Ht 31, and *dark shaded areas* indicate homology between Ht 21 and P150. *B*, helical wheel representation of RII-anchoring protein sequences. Each sequence was drawn as an α-helix with 3.6 amino acid residues/turn. *Shaded areas* identify the hydrophobic face of each amphipathic helix. Amino acid residues are represented by single-letter codes.

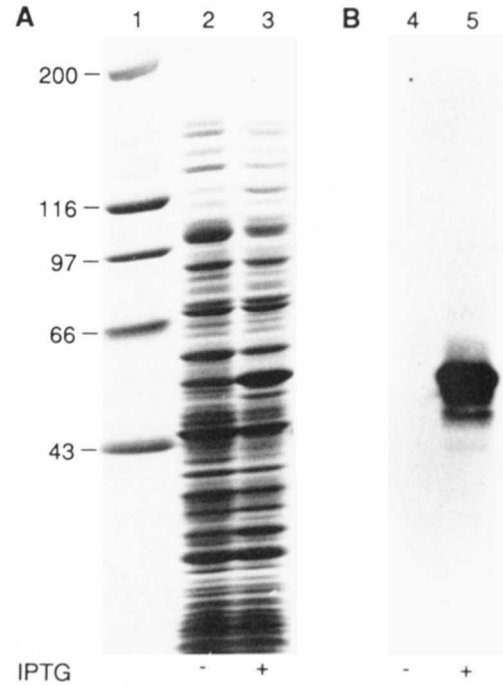

FIG. 2. **Binding of RII to expressed Ht 31 (418–736) fragment.** Bacterial extracts (50 μg of protein) were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. After separation, the proteins were electrophoretically transferred to Immobilon membranes for analysis by solid-phase RII binding assay. Comparison of Coomassie Brilliant Blue-stained blot (*A*) and autoradiograph (*B*) of control (*lanes 2* and *4*) or isopropyl-1-thio-β-D-galactopyranoside (*IPTG*)-induced (*lanes 3* and *5*) *E. coli* cells containing Ht 31 (418–736) in plasmid pET11d is shown; molecular mass markers are shown in *lane 1*. Extracts were analyzed for ^{32}P-labeled RIIα binding by a solid-phase overlay assay using a modified overlay procedure (19, 20).

ondary structure of the region and can result in a 20° bend in the helix. Hydrophobic residues, located within the second and third turns (helix residues 5 and 9) and at the COOH-terminal end of the predicted helix region (residue 14), were mutated to proline (Fig. 3*A*). Mutants were expressed in the pETkfc vector system, and each protein was analyzed for the ability to bind RII by solid-phase binding assay (Fig. 3, *B* and *C*). Furthermore, each mutant protein was assessed for RIIα interaction by gel-shift analysis in polyacrylamide gels (Fig. 4). This technique monitors protein-protein interactions under nondenaturing conditions and was performed to ensure that results obtained by solid-phase binding were not an artifact of the denaturing conditions used.

The introduction of proline into the amphipathic helix region of Ht 31 diminished or abolished RIIα binding (Figs. 3*C* and 4). Weak RIIα binding was observed with mutant Ht 31 Pro-498 (Fig. 3*C*, *lane 8*) which contained a mutation at position 5 in the putative helix region. No RIIα binding was obtained with either Ht 31 Pro-502 or Ht 31 Pro-507, which contained mutations at positions 9 and 14 (Fig. 3*C*, *lanes 9* and *10*, and Fig. 4). The double mutant, Ht 31 Pro-498/507, lacked RII-anchoring properties (Fig. 3*C*, *lane 12*, and Fig. 4) as did the triple mutant Ht 31 Pro-498/502/507 (data not shown). Proline substitution for Ala-522, which lies 12 residues downstream from the amphipathic helix region, had no apparent effect on RII binding (Fig. 3*C*, *lane 11*, and Fig. 4). These results suggest that disruption of protein secondary structure between residues 498 and 507 of Ht 31 diminishes or abolishes RII binding.

DISCUSSION

Computer-aided searches for potential secondary structure in four RII-anchoring proteins have identified a common region in each molecule which displays a high probability for amphipathic helix formation. The putative amphipathic helix in MAP 2 is located within the 31-amino acid RII-binding domain (24, 25), while the amphipathic helix region in P150 is located just upstream of the COOH-terminal region which, if removed, prevents binding to RII (19). The data presented in this study strongly suggest that a similar amphipathic helix in the thyroid RII-anchoring protein Ht 31 is required for binding RIIα. Mutations which should disrupt the helicity of this region prevent RIIα binding. These findings have led us to propose that amphipathic helices, on the surface of RII-anchoring proteins, form the sites for interaction with RIIα.

The amphipathic/amphiphilic α-helix is a common secondary structure motif found in several biologically active peptides and proteins (26). It is defined as an α-helix with opposing hydrophilic and hydrophobic faces orientated down the long axis of the helix. To test our hypothesis that an α-helix conformation between residues 494 and 507 of Ht 31 was required for interaction with RIIα, we disrupted the helicity of the region by substituting selected hydrophobic residues with proline. Proline is regarded as sterically incompatible with normal α-helical conformations because of the

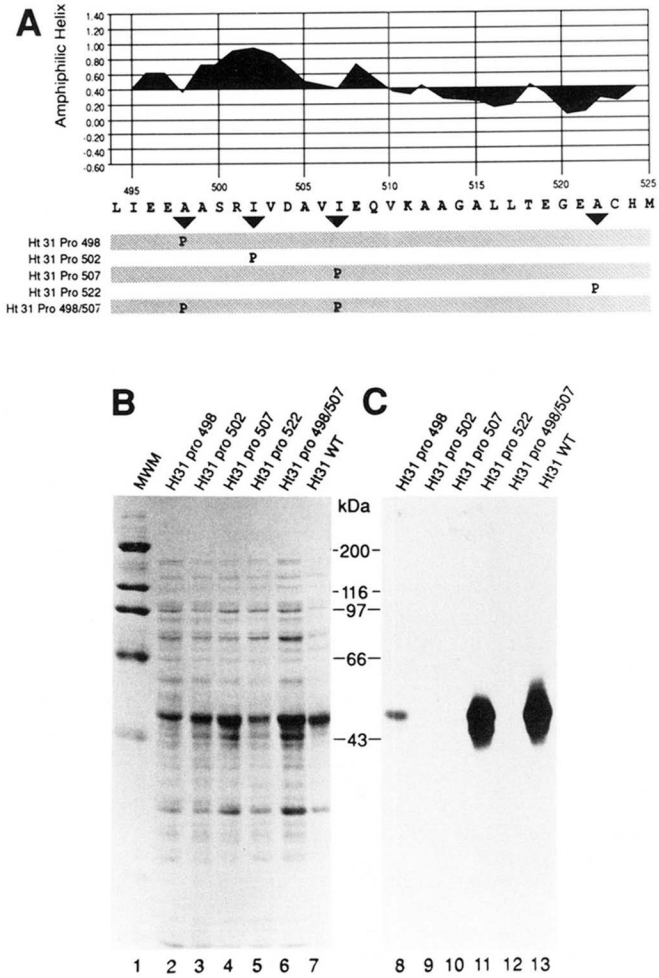

FIG. 3. **Disruption of the amphipathic helix region in Ht 31.** An amphipathic helix profile of amino acids 494–525 of Ht 31 was calculated according to the method Eisenberg and colleagues (30). *A*, shows the probability for amphipathic helix formation between residues 494 and 525. Values greater than 0.4 are considered positive indicators of amphipathic helix regions. Amino acid positions mutated to proline are indicated below. Point mutations were produced in the Ht 31 cDNA and expressed using the pETkfc plasmid. Mutant Ht 31 proteins were partially purified from bacterial extracts and separated by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and electrophoretically transferred to an Immobilon membrane. The blot was stained with Coomassie Brilliant Blue (*B*), and analyzed by autoradiography (*C*) after incubation with ^{32}P-labeled RIIα. *Lane 1* contains molecular mass markers (*MWM*) with the sizes indicated between the gels. Ht 31 wild type (*WT*) and the various proline mutations are in the indicated *lanes*.

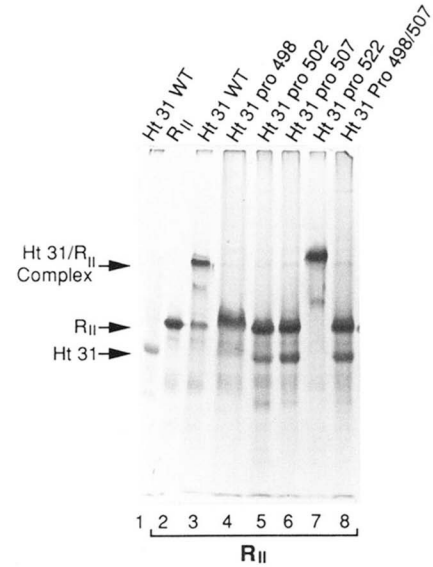

FIG. 4. **Binding of RIIα to Ht 31 and mutants under nondenaturing conditions.** The ability of Ht 31 and mutants to interact with RIIα was assessed by nondenaturing electrophoresis. Ht 31 mutants (5 μg) and RIIα (5 μg) were incubated for 1 h at 20 °C and separated by nondenaturing electrophoresis on a 6% (w/v) polyacrylamide gel. The RII-Ht 31 complex was identified as a band of slower mobility. *Lane 1* contains wild type (*WT*) Ht 31 alone; *lane 2*, RIIα alone; *lane 3*, Ht 31 wild type plus RIIα; *lane 4*, Ht 31 Pro-498 plus RIIα; *lane 5*, Ht 31 Pro-502 plus RIIα; *lane 6*, Ht 31 Pro-507 plus RIIα; *lane 7*, Ht 31 Pro-522 plus RIIα; *lane 8*, Ht 31 Pro-498/507 plus RIIα.

imino peptide linkage. For example, the recently solved crystal structure of the *E. coli* DNA-binding protein, FIS, contains a 20-residue α-helix which is distorted by 20° along its axis due to a proline at position 13 (31). Therefore, the substitution of proline at residues 498, 502, and 507 likely distorts the secondary structure at three distinct positions within the predicted α-helix, with only minor alterations to the amphipathic or hydrophobic nature of the region. Since each point mutation diminished or abolished RII binding, it is reasonable to assume that an α-helical conformation between residues 494 and 507 of Ht 31 is necessary for interaction with RII.

A characteristic of the amphipathic helix motif is the ordered placement of alternating pairs of hydrophobic and hydrophilic amino acids within the linear sequence of a protein (26). In addition to this criterion, each RII-anchoring protein contains acidic amino acids distributed over the hydrophilic face of the helix. In particular, an invariant glutamic acid at position 3 is located within the first turn of the amphipathic helix (Fig. 1*B*). Therefore, the overall sequence characteristics of an RII-anchoring site appear to be approximately a 14-residue region of sequence rich in acidic and hydrophobic amino acids with a high probability for amphipathic helix formation. The lack of a consensus RII binding sequence is consistent with the hypothesis that each molecule contains a common secondary structure. Another example of conserved secondary structure is the family of 20 or so calmodulin-binding peptides, which exhibit little or no conservation in primary structure other than an abundance of basic and hydrophobic amino acids (32). The basic faces of these amphipathic peptides form ionic interactions with the acidic central helix of calmodulin (32). In fact, synthetic peptides consisting of alternating pairs of leucine and lysine residues have nanomolar binding affinities for calmodulin (33). Future experiments are planned to synthesize RII-anchoring peptides and determine whether acidic residues within the hydrophilic face of the amphipathic helix region are essential for RII anchoring. The identification of the RII-anchoring site in Ht 31 has provided structural information which can be tested on Ht 21 and may allow the development of peptide antagonists of RII anchoring. These reagents will be introduced into cells in order to disrupt the normal subcellular localization of PKA *in vivo*.

Ht 31 and MAP 2 are 43% identical over the 14-residue amphipathic helix region, and Ht 21 and P150 have 93% sequence identity (Fig. 1*A*), suggesting that there may be two classes of RII-anchoring proteins. It is of interest to note that MAP 2 has a 7-fold higher affinity for the RIIα isoform than RIIβ (12). It will be important to establish whether Ht 21 and Ht 31 preferentially bind to a particular RII isoform. The differential subcellular distribution of RIIα and RIIβ in neurons, demonstrated by electron microscope immunocytochem-

istry (34), may reflect, in part, a preferential interaction with distinct RII-anchoring proteins. Structural and functional analysis of additional RII-anchoring proteins is required to establish if, in fact, there are two classes of RII-anchoring sites and if they preferentially associate with a specific RII isoform. Nevertheless, the preferential interaction of RII isoforms with specific classes of RII-anchoring proteins is an attractive mechanism to govern the subcellular localization of different PKA isoforms.

Our previous studies have shown that the RII dimer is required for anchoring protein interaction (22). Furthermore, we and others have shown that MAP 2 and a fragment of P75 (the bovine homolog of P150) bind at the same or overlapping sites on RII (22, 23). It is likely, therefore, that the amphipathic helix of the RII-anchoring protein interacts with sites on both RII subunits. The nature of these interactions is unclear but may involve ionic interactions between the acidic face of the amphipathic helix and basic residues located on both RII protomers. Whereas the amphipathic helix region is responsible for RII association, it is clear that other regions in each anchoring protein are responsible for specific interaction with membranes, organelles, or the cytoskeleton. This 2-fold binding could potentially determine the subcellular location of the PKA (6). Presumably, these interactions anchor PKA in close proximity to its preferred substrates (6). Recently, a splice variant of the bovine PKA catalytic subunit, Cβ2, has been identified which contains a putative amphipathic helix at the amino terminus, replacing the normal myristylation signal (35). The role of this amphipathic helix is unknown, but the authors suggest it may function to tether PKA catalytic subunit to membranes. Potentially, amphipathic helices could be responsible for anchoring both RII and Cβ2 subunits, such that the active catalytic subunit was immobilized and remained close to its regulatory subunit upon cAMP activation. Under these conditions, the accessibility of PKA to particular substrates would be tightly controlled and kinase activity would be highly regulated, since rapid reassociation of the holoenzyme complex would occur after cAMP concentrations returned to basal levels.

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