

Use of Synthetic Peptides in the Dissection of Protein-Targeting Interactions

John D. Scott and Maree C. Faux

1. Introduction

Phosphorylation of protein substrates by kinases and phosphatases is a major process in the control of cellular function (1). The mechanisms involved in the regulation of kinase and phosphatase activity have been the subject of intense investigation since glycogen phosphorylase was first recognized to be regulated by phosphorylation (2,3). The use of synthetic peptides has featured extensively in structure and function studies, establishing regions that are important for the control of substrate phosphorylation. The regulation of kinases and phosphatases is achieved at many levels. One level of regulation involves the subcellular localization of kinases and phosphatases through interactions with targeting proteins. This chapter will focus on the use of synthetic peptides in the identification of functional domains on targeting proteins, the characterization of bioactive peptides *in vitro*, and the use of peptides to disrupt enzyme localization in cells.

1.1. Kinase Substrates and Inhibitors

Knowledge of the substrate specificity of protein kinases has enabled an understanding of substrate and inhibitor recognition, which has shed some light on the way in which protein kinases are regulated (4). Synthetic peptide studies have proved to be very useful in determining specific side-chain determinants in a phosphorylation consensus sequence. These studies have demonstrated that synthetic peptides modeled on the phosphorylation site sequence of substrate proteins could be phosphorylated with kinetic constants comparable to natural substrates. For example, the cAMP-dependent protein kinase (PKA) phosphorylates the sequence LRRASLG (Kemptide) from the liver pyruvate

kinase with kinetic parameters similar to the native protein (5). The development of synthetic peptide substrates, such as Kemptide, has enabled selective and specific measurement of protein kinase activity in cell extracts, using the filter-paper assay of Corbin and Riemann (6) (see Subheading 3.3.2.1.). Since the introduction of Kemptide as the preferred commercial reagent to detect PKA activity, several peptide analogs have been developed to circumvent the need for radioactivity. In particular, fluorescent Kemptide analogs have been designed, and phosphorylation can be detected spectrophotometrically.

Many protein kinases exist in latent, inactive forms, because part of their structure blocks access to the active site. The pioneering work on this aspect of kinase regulation came from the laboratory of Corbin et al., where the autoinhibitory region of the type II regulatory (RII) subunit PKA was called a pseudosubstrate, because it has features that resemble the substrate and it binds in the active site (7). This hypothesis was largely confirmed by synthetic peptide studies performed in the laboratories of Kemp, Walsh, and Krebs, where pseudosubstrate sequences were shown to be potent competitive inhibitors of PKA activity (8–10). The type I regulatory (RI) subunit, as well as the heat-stable protein kinase inhibitor (PKI), was shown to inhibit PKA competitively with substrate (8,9,11). Structure–function studies with synthetic peptides of PKI(5–24) have demonstrated potent inhibition (2.3 nM) and identified the residues important for inhibition (8,9,11). Inhibitor peptides for other autoregulated protein kinases have also been identified and are listed in Table 1. The pseudosubstrate sequence of protein kinase C (PKC) is also a potent substrate antagonist that inhibits competitively, with respect to peptide substrate, and noncompetitively with ATP (12). Modification of peptides has enabled their use inside cells. For example, *N*-myristoylation of the peptide F²⁰ARKGALRQ²⁸, derived from the pseudosubstrate sequence of PKC, allows the peptide to permeate the cells and to specifically inhibit PKC (13). This method has recently been used to powerful effect in human breast cancer MCF7-MDR cells, in which the *N*-myristoylated peptide to the pseudosubstrate region of PKC- α has been shown to partially reverse multidrug resistance through inhibition of PKC- α (30). In addition, antipeptide antibodies raised against the pseudosubstrate sequence (residues 19–36) have been shown to activate PKC in the absence of external activators (31). The studies illustrate the many ways in which synthetic peptides have been used to understand aspects of structure and regulation of these enzymes, as well as their use as probes for biological function.

1.2. The Targeting Hypothesis

It is becoming apparent that the subcellular location of signaling enzymes represents an important means to control the actions of multifunctional protein kinases and phosphatases. A flurry of recent advances from a variety of labora-

Table 1
Protein Kinase Pseudosubstrate Peptide Inhibitors

| Protein kinase | Pseudosubstrate sequence | K _i /IC ₅₀ μM |
|--|---|-------------------------------------|
| cAMP-dependent PK PKI(5-24) | TTYADFIASGRTGRRNAIHD | 0.0023 |
| cGMP-dependent PK(α ₂) cGMP PKα(54-69) | GPRTTTRAQQISAEP | 2100 |
| Protein kinase C PKCβ(19-31) smMLCK | RFARKGALRQKNV | 0.13 |
| smMLCK(787-808) smMLCK(774-807) | SKDRMKKYMARRKWQKTGHAV LQKDTKNMEAKKLSKDRMKKYMARRKWQKTGHAV | 0.0012 0.0003 |
| CaM-II PK (α ₂) CaM-II PK(281-309) (290-309) | MHRQET*VDCLKKFNARRKLLKGAILTTMLA LKKFNARRKLLKGAILTTMLA | 2.7 24 |
| Phosphorylase β kinase (αβγδ) α(332-353) β(420-436) | VIRDPYALRPLRRLIDAYAFRI ³⁵³ KRNPQSQKRFPSNCGRD ⁴³⁶ | 921 |

Based on substrate and pseudosubstrate sequences described in ref. 35.

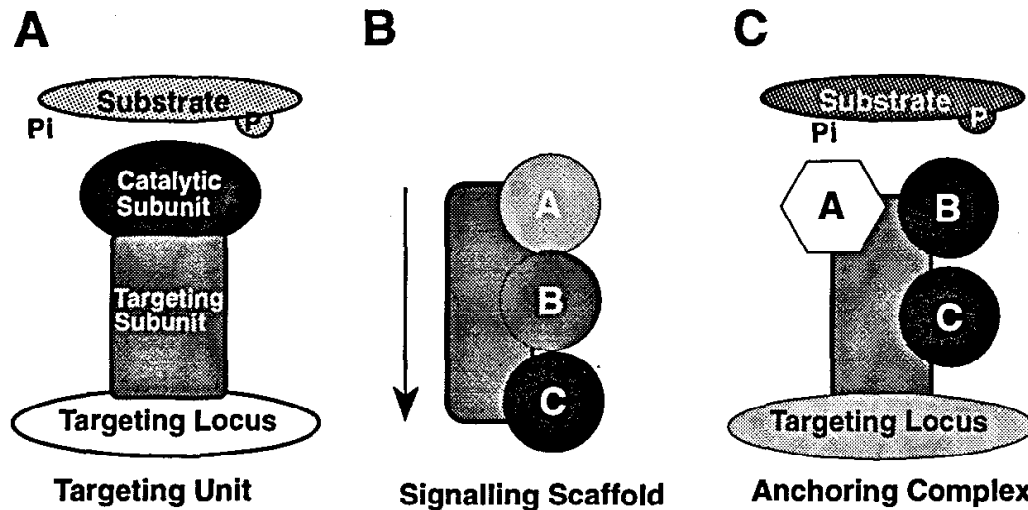


Fig. 1. Subcellular targeting of protein kinases and phosphatases. (A) Schematic diagram depicting the catalytic subunit of a kinase or phosphatase positioned close to substrate proteins through a targeting subunit. (B) Schematic diagram of a kinase signaling scaffold, where A, B, and C represent several kinases in a pathway bound to a scaffold protein. (C) Schematic diagram of an anchored kinase/phosphatase signaling complex, where A, B, and C represent kinases or phosphatases anchored close to substrate proteins.

tories indicates that the subcellular location of these enzymes is maintained by association with specific targeting proteins. For example, PKA, PKC, and the Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) are localized by specific binding proteins (17,23,25). Likewise, protein phosphatases (PP-) 1, 2A, and 2B are positioned through association with phosphatase targeting subunits. The "targeting hypothesis" proposes that targeting subunits or proteins specify the location and catalytic and regulatory properties of protein kinases and phosphatases (14). The targeting subunit is defined as that part of a kinase or phosphatase that directs the catalytic subunit to a subcellular location (Fig. 1A). An additional level of complexity in the subcellular targeting of signaling enzymes involves the formation of kinase-phosphatase signaling complexes, in which more than one enzyme may be targeted through one molecule. This allows the coordination of phosphorylation events through multivalent targeting proteins, providing an efficient means to regulate signal transduction events (15,32). Two related, but distinct, classes of targeting protein mediate this process. Scaffold proteins simultaneously associate with several kinases of a pathway, allowing signals to pass from one enzyme to the next (Fig. 1B); anchoring proteins are tethered to targeting loci and maintain their complement of enzymes close to their site of action (Fig. 1C). The following sections describe the use of peptides in defining the regions of A-kinase anchor proteins (AKAPs)

involved in the subcellular targeting of PKA, and their use as reagents to probe the function of anchored enzymes (*see Subheading 3.1.*). Chapter 5 describes methods used to study kinase anchoring using AKAPs as a model; this chapter will concentrate on our analysis of a neuronal anchoring protein, AKAP79, which has been shown to act as a scaffold for three signaling enzymes, PKA, PKC (*see Subheading 3.3.*), and PP-2B, calcineurin (CaN) (*see Subheading 3.2.*) (15).

2. Materials

2.1. Dissection of PKA–AKAP Interactions

2.1.1. Peptide Block of RII–AKAP Interaction in RII Overlay

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus and solutions.
2. Immobilon (Millipore, Bedford, MA) or nitrocellulose membrane (Protran, Schleicher & Schnell, Keene, NH).
3. BLOTTO: Tris-buffered saline, pH 7.0 (TBS), 5% dry milk, 1% bovine serum albumin (BSA).
4. RII (recombinant protein).
5. [γ - 32 P]-ATP (10 mCi/mL) NEN Dupont (Boston, MA).
6. PKA catalytic subunit (purified from bovine brain).
7. Reaction buffer: 50 mM MOPS, pH 6.8, 50 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mg/mL BSA.
8. Excellulose GF-5 desalting column (Pierce) (Rockford, IL).
9. Tris-buffered saline (TBS)/0.05% Tween-20 (TTBS).
10. Anchoring inhibitor peptides: Ht31 (493–509); control Ht31 (494–509Pro⁵⁰²), stock solutions stored at –20°C.
11. 3MM paper (Whatman) (Maidstone, UK).

2.1.2. Peptide Elution of AKAPs Following Copurification with RII

1. Bovine brain tissue.
2. Hypotonic buffer: 10 mM HEPES, pH 7.9, containing 1.5 mM MgCl₂, 10 mM KCl, 10 μ M IBMX, 0.5 mM DTT, and protease inhibitors: 1 mM AEBSF, 2 μ g/mL pepstatin/leupeptin, 1 mM benzamidine.
3. cAMP-agarose (Sigma, St. Louis, MO).
4. Anchoring inhibitor peptide and control (*see Subheading 2.1.1., step 10*).
5. 75 mM cAMP (Sigma).
6. 6% TCA.
7. SDS sample buffer: 80 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.0012% bromophenol blue.
8. SDS-PAGE apparatus and solutions.
9. 32 P-RII (prepared as described *Subheading 3.1.1.*).
10. Antibodies to RII.

2.1.3. PKA-Anchoring Inhibitor Peptides *in vivo*

1. Cultured rat hippocampal neurons (5–12 d).
2. Intracellular medium under recording conditions: 10 mM HEPES, pH 7.2, 65 mM NaCl, 2.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 1 μM tetrodotoxin, 100 μM picrotoxin.
3. Recording pipets containing: 10 mM HEPES, pH 7.3, 155 mM caesium gluconate, 10 mM BAPTA, 5 mM Mg-ATP, 2 mM MgCl₂.
4. Peptides: PKI (5–24), anchoring inhibitor peptides: Ht31(493–509) and AKAP79(394–409), control anchoring inhibitor proline peptide Ht31(493–509Pro⁵⁰²).
5. PKA catalytic subunit.

2.1.4. Delivery of Cell Soluble Peptide

1. *N*-myristoylated anchoring inhibitor peptide.
2. Nonmyristoylated anchoring inhibitor peptide.
3. Cultured rat hippocampal neurons.
4. 0.1% DMSO/phosphate-buffered saline (PBS).
5. PBS.
6. 3.7% Formaldehyde in PBS.
7. 100% Acetone stored at –20°C; PBS containing 0.1% BSA stored at 4°C.
8. Peptide antibody to anchoring inhibitor peptide (stored at 4°C).
9. Fluorescent secondary antibody (Molecular Probes, Eugene, OR).

2.2. Dissection of Calcineurin–AKAP Interactions

2.2.1. CaN Activity Assay

2.2.1.1. PREPARATION OF ³²P-RII PEPTIDE SUBSTRATE

1. [γ-³²P]-ATP; radioactive concentration 10 mCi/mL (NEN Dupont).
2. “Cold” ATP 10 mM stock, pH 7.0 (stock aliquoted and stored at –20°C).
3. pH 1.9 buffer: 2% formic acid, 8% acetic acid, 90% H₂O.
4. RII peptide.
5. PKA catalytic subunit (2 μg/mL).
6. [γ-³²P]-ATP stock (1000 cpm/pmol).
7. Reaction buffer: 20 mM MOPS, pH 7.0, 2 mM MgAcetate, 5 mM β-mercaptoethanol.
8. Acetic acid.
9. AG1X8 resin (Bio-Rad [Hercules, CA]).
10. C-18 Sep Pak cartridge (Pierce).
11. 0.1% TFA.
12. 50% CH₃CN, 49.9% H₂O, 0.1% TFA.
13. 1M (NH₄)₂CO₃.
14. pH paper.

2.2.1.2. CaN ASSAY

1. 5X concentrate assay buffer: 200 μM Tris-HCl, pH 7.5, 0.5M KCl, 0.5 mM CaCl₂, 30 mM MgAcetate, 2.5 mM DTT, and 0.5 mg/mL BSA, stored at 4°C; DTT added freshly from 1M stock stored at –20°C.

2. Calmodulin 5 mg/mL stock.
3. CaN (recombinant protein expressed in baculovirus).
4. CaN diluting buffer: 40 μ M Tris-HCl, pH 7.5, 0.1M KCl and 0.5 mM DTT, stored at -20°C .
5. ^{32}P -RII peptide (prepared as described in Subheading 3.2.1.1.)
6. 75 mM phosphoric acid.
7. AG50W-X8 resin (Bio-Rad).
8. Glass Pasteur pipets with glass bead placed at base to make a column or polyprep plastic columns (Bio-Rad).
9. Aqueous-compatible scintillant.

2.2.2. Inhibition of CaN

As for CaN assays (see Subheading 2.2.1.2.); CaN inhibitor peptide AKAP79(88–102).

2.3. Dissection of PKC–AKAP Interactions

2.3.1. Peptide Block of PKC–AKAP Interaction in PKC Overlay

1. SDS-PAGE apparatus and solutions.
2. Immobilon or nitrocellulose membrane.
3. BLOTTO: TBS, pH 7.0, 5% dry milk, 1% BSA.
4. TBS, pH 7.0.
5. Phosphatidylserine (PS) 20 μ g/mL: Brain extract Type III from Sigma.
6. 1.4 mM PS/0.04 mM diacylglycerol (DG) in 20 mM HEPES, pH 7.5 (see Note 23), from Avanti Polar Lipids (Alabaster, AL).
7. PKC: Partially purified from rabbit brain.
8. PKC assay buffer: TBS, 1% albumin, 1.2 mM Ca^{2+} , 1 mM EGTA, 10 μ g/mL leupeptin, 10 μ g/mL pepstatin.
9. PBS wash buffer: PBS, pH 7.0, 1.2 mM Ca^{2+} , 1 mM EGTA, 20 μ g/mL PS.
10. 3.7% Paraformaldehyde/PBS stock.
11. PBS + 2% glycine.
12. AKAP peptides (residues 31–52 from AKAP79 to block PKC binding; AKAP79 388–409 as control RII binding peptide).
13. Chemiluminescence reagents (Pierce).

2.3.2. Peptide Inhibition of PKC Activity

2.3.2.1. PKC ACTIVITY ASSAYS

1. 4X concentrate assay buffer stock: 80 mM HEPES, pH 7.5, 40 mM MgCl_2 , 4 mM DTT.
2. 10X stocks of the following effectors: 3 mM Ca^{2+} (or 5 mM EGTA); 1.4 mM PS/0.04 mM DG in 20 mM HEPES, pH 7.5 (see Note 23) (or 20 mM HEPES, pH 7.5).
3. 1 mM [γ - ^{32}P]-ATP (200 cpm-pmol).
4. EGF-Receptor peptide substrate (Sigma), stock concentration 5 mg/mL (\sim 4.175 mM), diluted to 10 μ M in assay.
5. PKC β II (expressed in baculovirus), diluted to 5 nM (final) in assay.

6. PKC diluting buffer: 20 mM Tris-HCl, pH 7.9, 1 mg/mL BSA, 1 mM DTT.
7. Phosphocellulose P81 paper (2 × 2-cm squares).
8. Wire cage that fits inside 500-mL beaker.
9. 75 mM Phosphoric acid.
10. Hair dryer used to dry P81 papers.

2.3.2.2. INHIBITION OF PKC

1. As for PKC assay (*see Subheading 2.3.2.1.*).
2. Peptide inhibitor AKAP79 (residues 31–52).

3. Methods: Dissection of Signaling Complexes with Bioactive Peptides

3.1. Dissection of PKA–AKAP interactions

Since several AKAPs apparently bind to the same or overlapping sites on RII α , it seemed likely that these molecules share a common RII-binding domain. However, comparison of these sequences revealed no striking homology (**Fig. 2A**), leading us to examine the RII-binding site in each anchoring protein for a conserved secondary structure binding motif. Computer-aided secondary structure predictions of each putative RII-binding site showed a high probability for amphipathic helix formation. The distinction between the hydrophobic and hydrophilic faces can be clearly seen when the sequences are drawn in a helical-wheel configuration (**Fig. 2B**). In each RII-anchoring protein there was a similar alignment of acidic residues throughout the hydrophilic face of each putative helix.

Analysis of Ht 31, a novel human thyroid RII-anchoring protein of 1035 amino acids, identified a potential amphipathic helix between residues 494 and 509 (**16**). This sequence (Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Gln) was 43% identical to region within the RII-binding site of MAP 2 (**Fig. 2A**). A peptide that spans the putative amphipathic helix region of Ht 31 binds RII α with an affinity of approx 4 nM (**18**), and circular dichroism analysis suggests it can adopt an α -helical conformation. This anchoring-inhibitor peptide has been used extensively as a tool to probe for AKAP-RII interaction in solid-phase RII overlays (*see Subheading 3.1.1.*) and to specifically elute AKAPs following copurification with RII (*see Subheading 3.1.2.*). An additional method for dissecting PKA–AKAP interactions has involved the use of biotinylated anchoring inhibitor peptides in a peptide overlay technique. This method is described in detail in Chapter 5 of this volume. The anchoring-inhibitor peptide has recently been used to disrupt the interaction between RII and AKAPs in neurons, which affects the modulation of glutamate receptor channels (*see Subheading 3.1.3.*). Finally, *N*-myristoylation of this peptide allows it to be permeable to cells (*see Subheading 3.1.4.*).

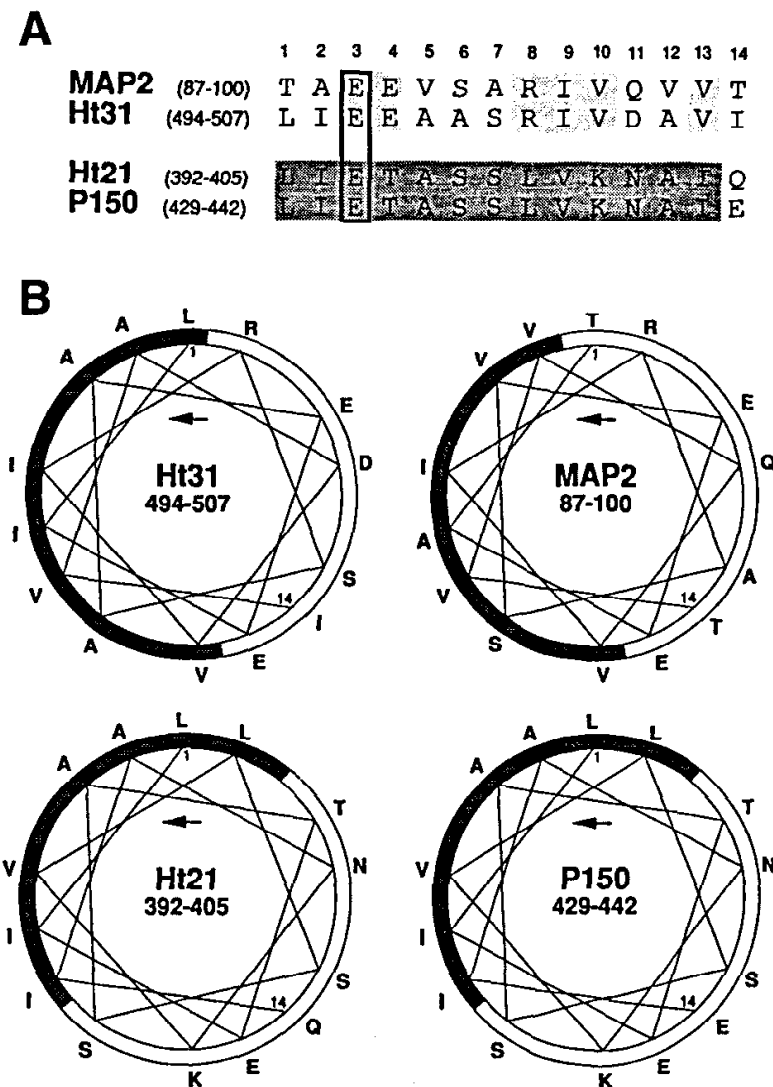


Fig. 2. The RII-binding region of AKAPs. (A) Primary sequence comparison of the RII binding region of 4 AKAPs, MAP2, Ht31, Ht21 (AKAP79), and P150 (AKAP150). (B) Helical wheel representation of the four AKAPs in (A) drawn as an amphipathic helix. The shaded area indicates hydrophobic residues and the open area indicates hydrophilic residues. Amino acids are indicated in the single letter code. The arrow indicates the direction of the helix.

3.1.1. Peptide Block of RII-AKAP Interaction in RII Overlay

The RII overlay is an established method for detection of RII-binding proteins (such as AKAPs) (17). The anchoring-inhibitor peptide is incubated with radiolabeled RII and specifically blocks interaction of the protein immobilized on the blot and the radiolabeled RII. A control peptide for the Ht31 peptide (residues 493-509) has been designed that has a single proline substituted for isoleucine at 502, which disrupts the amphipathic helix and can no longer bind RII.

1. Separate protein samples by SDS-PAGE and transfer to Immobilon or nitrocellulose membranes by standard electrotransfer techniques (**ref. 33**; *see Note 1*).
2. Block membrane by incubation with BLOTTO + 1% BSA (TBS, pH 7.0, 5% dry milk, 1% BSA) for 1 h at room temperature (*see Note 2*).
3. RII protein (2 μg) is radiolabeled by incubation with PKA catalytic subunit (0.1 μg) and [γ - ^{32}P]-ATP (50 μCi) in a reaction buffer containing 50 mM MOPS, pH 6.8, 50 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 0.1 mg/mL BSA at 30°C for 15 min.
4. Separate radiolabeled protein from free ^{32}P -ATP on a cellulose GF-5 desalting column equilibrated in TBS/0/05% Tween-20 (TTBS) (*see Note 3*).
5. Incubate anchoring inhibitor peptide (e.g., Ht31 [residues 493–509] peptide or control proline peptide Ht31 peptide [residues 493–509 Pro⁵⁰²] [0.4–1.0 μM] and radiolabeled RII (specific activity 10⁵ cpm/mL) in BLOTTO with blocked-protein blot, for a minimum of 4 h at room temperature, with agitation (*see Notes 4 and 5*).
6. Wash blot extensively with TTBS (4 \times 15 min) to remove free ^{32}P -RII.
7. Expose to film overnight at -70°C and develop autoradiograph.

3.1.2. Peptide Elution of AKAPs Following Copurification with RII

A common method to demonstrate that a complex between an AKAP and RII exists inside cells is to copurify the complex using an affinity matrix, such as cAMP agarose. A cell lysate is passed over cAMP-agarose and nonspecific binding proteins removed before specific elution with cAMP. A variation on this technique is to elute the AKAP from RII using the anchoring-inhibitor peptide (**Fig. 3B**), followed by the cAMP elution to remove the RII from the cAMP agarose (**Fig. 3C**). Thus, the interaction of the AKAP with RII is shown to specifically occur through an amphipathic helix on the AKAP, which is disrupted in the presence of the anchoring-inhibitor peptide. CaN also copurified with RII and the AKAP, and was shown to be displaced with the AKAP from RII (**Fig. 3A**) (*see Subheading 3.2.*).

1. Prepare cell lysate from tissue or cultured cells. For example, for copurification of RII and AKAP75 (the bovine homolog of AKAP79), a bovine brain extract was prepared from flash-frozen bovine cortex. Chunks (approx 3 g) of frozen brain cortex were ground to a fine powder in liquid nitrogen with a mortar and pestle. The frozen powder was added to 6 mL ice-cold hypotonic buffer (10 mM HEPES, pH 7.9 containing 1.5 mM Mg Cl₂, 10 mM KCl, 10 μM IBMX, 0.5 mM DTT, 0.1% NP40, and protease inhibitors: 1 mM AEBSF, 2 $\mu\text{g}/\text{mL}$ pepstatin/leupeptin, 1 mM benzamidine). The frozen slurry was homogenized until uniform in consistency in several batches with an ice-cold dounce homogenizer. The homogenate was centrifuged at 25,000g for 30 min at 4°C and the lysate supernatant retained.
2. Incubate lysate supernatant (2 mL) with cAMP-agarose (1 mL packed beads) overnight at 4°C on a horizontal rotator.
3. Centrifuge at 3000g for 5 min at 4°C and decant supernatant.

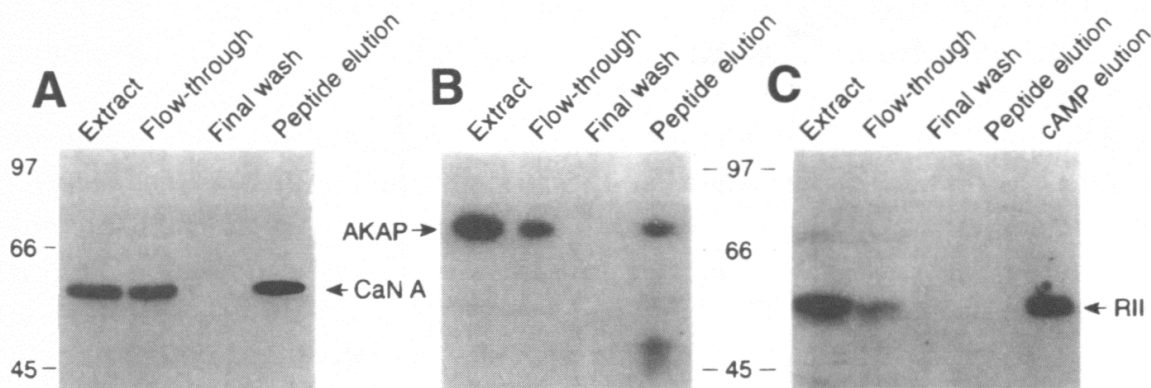


Fig. 3. Copurification of CaN and PKA from bovine brain by affinity chromatography on cAMP-agarose. The Ht31 (493–515) peptide was used to specifically displace AKAPs from the cAMP-affinity column. Protein blots were probed with antibodies to CaN (A), ^{32}P -RII to detect AKAPs (B), or antibodies to RII (C). The latter shows that RII was still bound to the cAMP-agarose following peptide elution, but was specifically eluted with cAMP. Positions of mol-wt markers are indicated in kDa (23).

4. Resuspend the cAMP-agarose beads in hypotonic buffer and transfer to a 2-mL column (Bio-Rad).
5. Wash column with 5 column volumes hypotonic buffer, containing 1M NaCl, and 20–30 column volumes hypotonic buffer (see Note 6).
6. Elute the AKAP from RII with 1 ml 0.5 mM anchoring-inhibitor peptide or the control proline peptide (see Subheading 3.1.1.) by incubating with cAMP-agarose beads at room temperature for 1 h.
7. Elute RII from the cAMP-agarose with 1 mL 75 mM cAMP by incubation at room temperature for 1 h (see Notes 7 and 8).
8. Concentrate the 1M NaCl wash, low-salt wash, peptide, and cAMP elutions by TCA precipitation in order to load all of the sample on a SDS-gel. Add ice-cold TCA to 6% to each sample and incubate on ice for 20–30 min. Centrifuge at 16,000g for 5 min and remove supernatant. Wash protein pellet with ether/ethanol (80:20), vortex vigorously, and centrifuge at 16,000g. Remove supernatant. Allow pellet to dry before adding SDS-sample buffer, boil for 5 min, and load on SDS-gel (see Note 9).
9. Detect proteins by RII overlay and/or Western blot (see Fig. 3).

Comment: This technique can also be used for immunoprecipitation experiments, in which an antibody to an AKAP is used to precipitate the AKAP and the proteins that are associated with it. To demonstrate that RII is specifically associated, the anchoring inhibitor peptide is used to displace RII from the AKAP. This technique has tremendous advantages over the more general SDS-sample buffer elution.

3.1.3. PKA-Anchoring Inhibitor Peptides In Vivo

The majority of RII/AKAP interactions have been studied in vitro and under nonphysiological conditions. However, the high affinity of the amphipathic

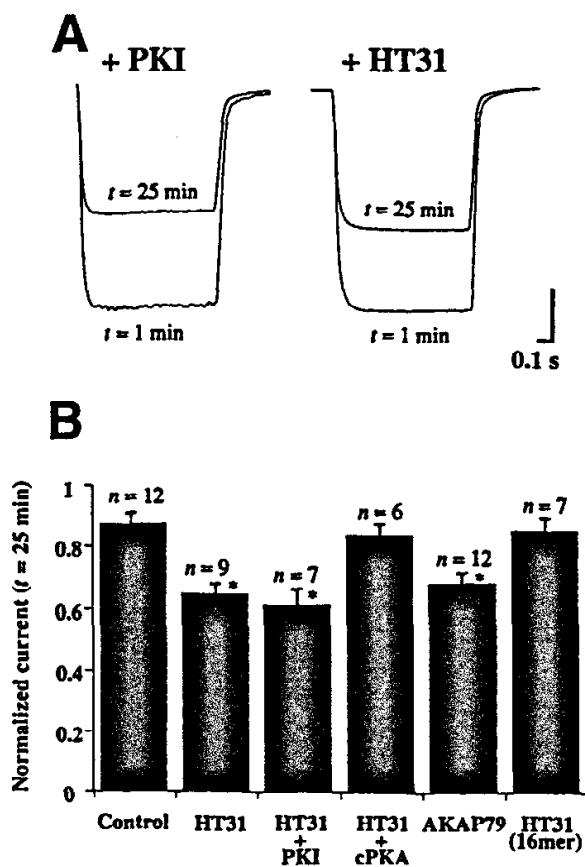


Fig. 4. Displacement of RII by anchoring inhibitor peptides blocks the regulation of AMPA/kainate channels by PKA. (A) Inward currents evoked by kainate ($20 \mu\text{M}$) at 1 and 25 min after the start of whole cell recordings are superimposed. Left, in presence of PKI(5–24) ($1 \mu\text{M}$) in the recording pipet. Right, in presence of the Ht31 anchoring-inhibitor peptide. (B) Amplitude of AMPA/kainate currents after 25 min of recording in the presence of ATP in the patch pipet. Histogram bars show mean current amplitudes (20).

helix peptides for RII made them ideal antagonists of PKA anchoring in vivo. The test system for these studies was the compartmentalization of PKA to the postsynaptic densities in hippocampal neurons, in which the kinase has easy access to the ionotropic glutamate receptors. These receptors are central to the process of signal transduction across the synaptic membranes; PKA-dependent phosphorylation is required to maintain the activity of AMPA/kainate responsive glutamate receptor channels (19,20).

Bioactive peptides were introduced into the neurons via a microdialysis technique that took advantage of the patch pipet as a delivery system. The role of PKA in maintaining channel activity was confirmed by a gradual decline in whole-cell currents evoked by kainate ($20 \mu\text{M}$), recorded in the presence of ATP ($20 \mu\text{M}$) and $1 \mu\text{M}$ PKI (5–24) peptide, a potent and specific inhibitor of the catalytic subunit of PKA ($61.8 \pm 3.2\%$ $n = 11$) (Fig. 4A). To test the role

of AKAPs in localizing the kinase near the channel, the anchoring-inhibitor peptides ($1 \mu\text{M}$) were added to the whole-cell pipet. The anchoring-inhibitor peptide derived from two AKAPs, Ht 31 or AKAP79, inhibited AMPA/kainate currents to the same extent as the PKI peptide ($64.9 \pm 3.2\%$ $n = 12$ and $68.8 \pm 3.3\%$ $n = 12$) (Fig. 4B). The effects of PKI and the anchoring inhibitor peptides were not additive. However, the action of the Ht 31 peptide could be overcome by the C subunit of PKA ($0.3 \mu\text{M}$) suggesting that the anchoring inhibitor peptide interfered with PKA-dependent phosphorylation, but did not directly inhibit the kinase. In addition, the control peptide unable to block RII/AKAP interaction had no effect on kainate currents ($85 \pm 4.1\%$ $n = 7$). Finally, currents evoked by AMPA ($1 \mu\text{M}$ $n = 6$) behaved in the same manner as those evoked by application of kainate. These results indicate that PKA localization is required for modulation of AMPA/kainate currents. These studies represented the first physiological evidence of the importance of PKA anchoring in the modulation of a specific cAMP-responsive event (20). Recently, Catterall and colleagues have used these peptides to demonstrate that disruption of PKA anchoring close to the L-type Ca^{2+} channel is required to maintain the channel in the active state (21). The functional effect of phosphorylation of the receptors appears to be desensitization for their agonist.

3.1.4. Delivery of Cell-Soluble Peptides

In order to perform the biochemical experiments, it is often necessary to introduce bioactive peptides into cells. Recently, covalently modified forms of peptides have been introduced into fibroblasts and neurons. A myristoylated peptide based on the Ht31(493–515) anchoring inhibitor peptide was synthesized according to the methods of Eichholtz et al. (13). This technique demonstrates that myristoylation is a viable means to introduce these peptides into living cells.

1. Dissolve myristoylated peptide and nonmyristoylated peptide 10 mM (anchoring-inhibitor peptide) in 100% DMSO and dilute to a final concentration of $500 \mu\text{M}$ in 5% DMSO/PBS (see Note 10).
2. Incubate myristoylated and nonmyristoylated control peptide with living cultures of rat hippocampal neurons for 1 h at 37°C (see Note 11).
3. Wash cultures extensively in PBS.
4. Fix cultures with 3.7% formaldehyde for 5 min at room temperature, wash in 100% acetone at -20°C for 1 min and incubate in a blocking solution of PBS containing 0.1% BSA for 30 min at room temperature.
5. Stain individual cover slips for uptake of myristoylated peptide with an antibody to the Ht31 (493–509) peptide followed with fluorescently labeled goat antirabbit IgG secondary antibody (see Note 12). Alternatively, the myristoylated peptide can be labeled with a fluorescent tag and intracellular uptake visualized directly by fluorescence.

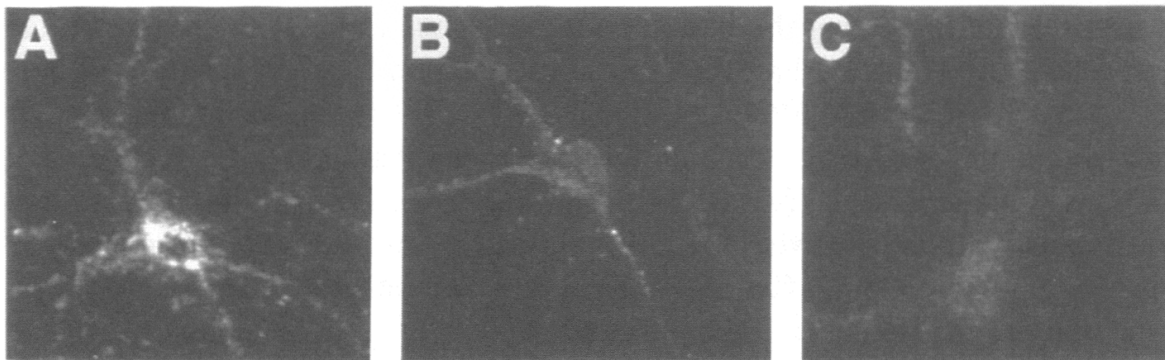


Fig. 5. Confocal immunomicroscopy of cultured rat hippocampal neurons incubated with myristoylated anchoring inhibitor peptide (**A**), nonmyristoylated anchoring inhibitor peptide (**B**), or absence of peptide (**C**).

6. Indirect immunofluorescent detection of intracellular uptake of peptide visualized using a confocal microscope. The uptake of the myristoylated anchoring-inhibitor peptide is shown in **Fig. 5A**. Confocal analysis of individual neurons was performed on nine focal planes ($0.5\ \mu\text{m}$) to confirm that detection of immunofluorescence was predominantly intracellular. Increased amounts of the myristoylated Ht31 (493–515) peptide were detected inside neurons (**Fig. 5A**) when compared to cells incubated with the non-myristoylated form (**Fig. 5B**). Control experiments incubated in the absence of peptide demonstrate the background level of staining (**Fig. 5C**).

3.2. Dissection of Calcineurin–AKAP Interactions

Bioactive peptides have also been used as reagents to decipher the complex web of protein–protein interactions that participate in the formation of the AKAP79 signaling complex. This section outlines studies that utilized synthetic peptides to map binding sites on AKAP79 for the protein phosphatase-2B, calcineurin (CaN). Our model for colocalization of PKA and CaN through AKAP79 implies that the AKAP contains distinct sites for kinase and phosphatase binding (**23**). Residues 88–102 of AKAP79 (Arg-Arg-Lys-Arg-Ser-Glu-Ser-Ser-Lys-Gln-Gln-Lys-Pro-Phe-Lys) were considered likely to comprise the CaN binding site because of homology with a region of the immunophilin FKBP-12 that contains determinants for CaN association (**Fig. 6A**). As CaN was inactive when isolated as a complex with the AKAP, we examined the effects of a synthetic peptide, corresponding to AKAP79 residues 81–102, on CaN activity. The peptide inhibited both CaN forms, but the Ht31 (493–515) peptide did not inhibit CaN (**Fig. 6B**). In addition, the observed inhibition was specific for calcineurin; the peptide did not significantly affect the activity of protein phosphatases 1 or 2A, at peptide concentrations as high as $0.4\ \text{mM}$. The following outlines the methods used to assay CaN and measure the inhibition caused by the AKAP79 peptide (residues 81–802).

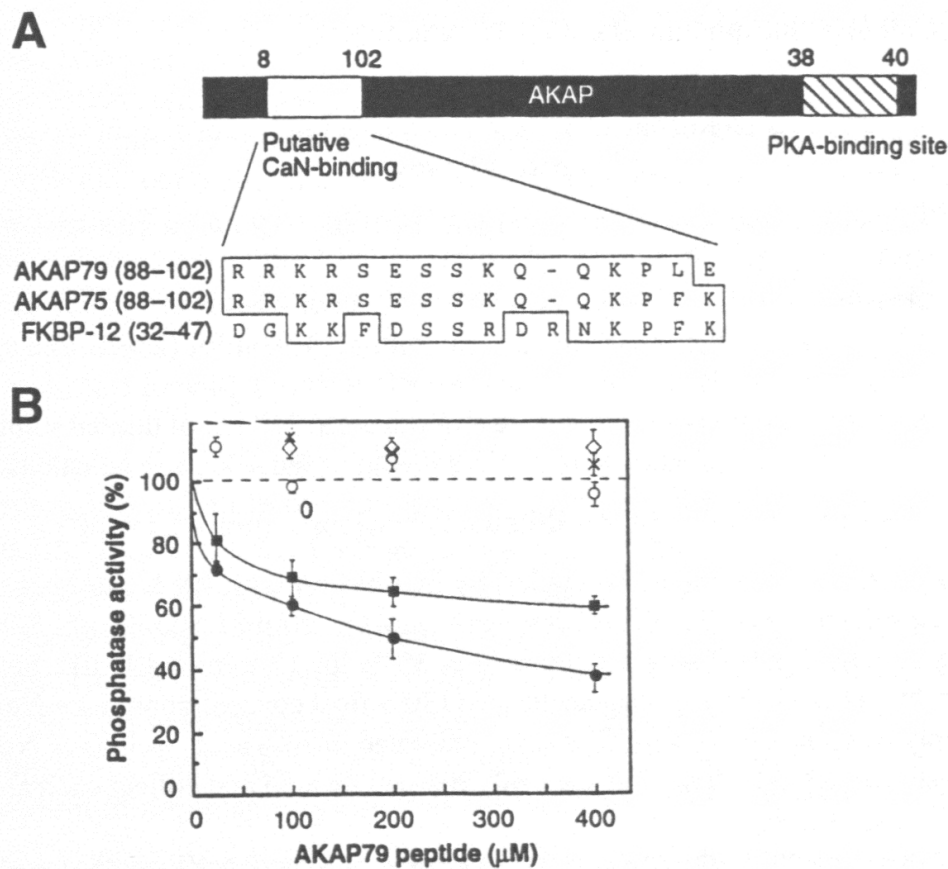


Fig. 6. Inhibition of CaN by AKAP79(88–102) peptide. (A) Schematic representation of AKAP79, indicating the PKA- and CaN-binding sites. Also shown is the sequence comparison of sites within AKAP79, AKAP75, and bovine FKBP12. Amino acid numbers are indicated and residues are indicated by the single letter code. (B) Dose–response curves of CaN (filled circles) and the Ca²⁺-calmodulin independent fragment of CaN (CaN₄₂₀) (filled squares) activity in the presence of the AKAP79(88–102) peptide. A control peptide corresponding to the RII-binding site of Ht31 did not inhibit CaN activity (open circles). The AKAP79 peptide did not inhibit protein phosphatase 1 (open diamonds) or 2A (crosses). Values are mean ± SD (23).

3.2.1. CaN Activity Assay

The substrate used to assay CaN activity is a phosphorylated peptide derived from RII (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu).

3.2.1.1. PREPARATION OF ³²P-RII PEPTIDE SUBSTRATE

1. To make [γ -³²P]-ATP stock (34):

a. Calculate the amount of [γ -³²P]-ATP required: (for a 1-mL final volume)

$$\begin{aligned} & \text{desired specific activity (cpm/pmol)} \times \text{conc. (mmol/L)} \times 10^6 / \\ & [\text{radioactive concentration of material as supplied (mCi/mL)} \times 2.2 \times 10^6] \\ & = \text{volume } [\gamma\text{-}^{32}\text{P}]\text{-ATP } (\mu\text{L}) \text{ to be added} \end{aligned}$$

- b. Calculate the amount of cold ATP required:

$$\text{volume cold ATP} = \frac{\text{desired conc. ATP} \times \text{total volume (e.g., 1 mL)}}{\text{conc. ATP stock}}$$

- c. Add calculated volume of [γ - ^{32}P]-ATP, cold ATP and adjust to 1 mL with H_2O . (see Note 13).
- d. Measure specific activity of [γ - ^{32}P]-ATP stock: Take an aliquot of [γ - ^{32}P]-ATP stock (10 μL) and dilute to 1 mL with pH 1.9 buffer (2% formic acid: 8% acetic acid:90% H_2O). The extinction coefficient of a 1 mmol/L stock of ATP at pH 1.9 at 257 nm is 14.7. Measure absorbance at 257 nm of diluted solution and count 10- μL aliquots. Specific activity (SA) of the stock can be calculated:

$$\text{SA (cpm/pmol)} = \text{cpm} \times \text{dilution factor} / [\text{conc. } (\mu\text{mol/L}) \times 10^6]$$

2. RII peptide (1 mM) is radiolabeled by incubation with PKA catalytic subunit (2 $\mu\text{g/mL}$) and [γ - ^{32}P]-ATP (1000 cpm/pmol) in 20 mM MOPS, pH 7.0, 2 mM MgAcetate, 5 mM β -mercaptoethanol at 30°C for 15 min in 400 μL . Stop reaction by adding 120 μL glacial acetic acid (30% final concentration) (see Note 14).
3. Apply reaction mix to AG1X8 resin equilibrated in 30% acetic acid (see Note 15).
4. Wash the AG1X8 resin with 4 \times 1 mL 30% acetic acid and collect each aliquot in microfuge tubes.
5. Dry down samples eluted from AG1X8 resin in Speedvac (Sorvell).
6. Resuspend dried samples in 0.1% TFA (500 μL) and apply to activated C-18 Sep Pak cartridge (see Note 16).
7. Apply radiolabeled RII peptide to cartridge and collect flow through. Wash with 0.1% TFA (10 mL) and collect flow through. Elute ^{32}P -RII peptide with 50% CH_3CN , 0.1% TFA (5 \times 1 mL) and collect in microfuge tubes (5 \times 1 mL). Count Cerenkov radiation for sample, flow through and each 1 mL elution (see Note 17).
8. Dry down eluted ^{32}P -RII peptide and resuspend in 500 μL H_2O . Add 1M NH_4CO_3 to neutralize (check with pH paper).
9. Dry down sample and resuspend ^{32}P -RII peptide in 200 μL H_2O . Count Cerenkov radiation (2 μL) (see Notes 18 and 19).

3.2.1.2. CAN ASSAY

Protein phosphatase assay using AG50W-X8 resin to separate ^{32}P -RII from free phosphate. The AG50W-X8 is a cation-exchange resin that binds the phosphopeptide; the released $^{32}\text{P}_i$ passes through directly into the scintillation vial. This involves pouring 1-mL AG50W-X8 columns for each reaction tube.

1. Make a stock containing 5X concentrate assay buffer. The final concentration in the assay should be: 40 μM Tris-HCl, pH 7.5, 0.1M KCl, 0.1 mM CaCl_2 , 6 mM MgAcetate, 0.5 mM DTT, and 0.1 mg/mL BSA.
2. In a 20- μL reaction volume, add 5X assay buffer, 1.5 μM calmodulin (final conc.) and 1 μM CaN (final conc.) diluted in ice cold buffer containing 40 μM Tris-HCl, pH 7.5, 0.1M KCl, and 0.5 mM DTT, to each tube. Preincubate tubes at 30°C (see Note 20).

3. At 20-s intervals, start reaction with addition of ^{32}P -RII peptide (30 μM), and vortex (*see Note 21*).
4. Stop reaction with 75 mM phosphoric acid (100 μL) at 20-s intervals.
5. When all time-points have been taken, add the 120- μL reaction volume to 1-mL AG50W-X8 columns, equilibrated in H_2O , and placed in scintillation counting vials. This allows direct collection of the free phosphate released following dephosphorylation of the ^{32}P -RII peptide by the phosphatase (*see Note 22*).
6. Wash AG50W-X8 columns with $4 \times 250 \mu\text{L}$ H_2O , add aqueous scintillant, and count by liquid scintillation counting.

3.2.2. Inhibition of CaN

To determine the half-maximal inhibitor constant for a peptide, assay CaN as described above, but in the presence of increasing amounts of inhibitor peptide. It is important to assay CaN activity at K_m for the ^{32}P -RII peptide. The K_m can be determined by assaying CaN over a range of substrate (^{32}P -RII) concentrations and plotting a double-reciprocal plot of $1/v$ ($\mu\text{mol}/\text{min}/\text{mg}$) vs $1/\text{substrate conc.}$ (μM) (Lineweaver-Burk plot). The K_m is then determined from the x -intercept. The half-maximal inhibition of a peptide inhibitor is determined by plotting the percent CaN activity vs inhibitor concentration. 100% CaN activity is the amount of dephosphorylation of the substrate in the absence of inhibitor.

3.3. Dissection of PKC–AKAP Interactions

PKC, a family of ser-thr kinases, is tethered to the postsynaptic density (PSD) through association with binding proteins (26). We used a solid-phase binding assay (overlays) (*see Subheading 3.3.1.*) to demonstrate recombinant AKAP79 bound to PKC in the presence of Ca^{2+} and phosphatidylserine. Fragments encompassing the first 75 residues of AKAP79 bound PKC, but COOH-terminal fragments containing the RII and CaN-binding regions did not, which implies that PKC binds to AKAP79 at a site that is distinct from those bound by RII and CaN (27,28). Basic and hydrophobic regions are determinants for binding of certain proteins to PKC (15), which drew our attention to a region located between residues 31 and 52 of AKAP79 (Fig. 7A). A peptide encompassing this region (Lys-Ala-Ser-Met-Leu-Cys-Phe-Lys-Arg-Arg-Ly-Lys-Ala-Ala-Lys-Ala-Pro-Lys-Ala-Gly) specifically blocked the interaction of AKAP79 with PKC in the overlay assay (Fig. 7B) (*see Subheading 3.3.1.*), but did not affect RII binding to the AKAP (Fig. 7C). Conversely, the RII anchoring inhibitor peptide (AKAP79 390–411) did not affect PKC-binding (Fig. 7B), but did block interaction with RII (Fig. 7C). We had observed previously that many kinases or phosphatases bind to anchoring proteins in an inactive state (22,23). Accordingly, recombinant AKAP79 protein inhibited PKC activity ($\text{IC}_{50} = 0.35 \pm 0.06 \mu\text{M}$, $n = 3$) (Fig. 7D). In addition, the AKAP79 peptide (residues 31–52) and a recombinant AKAP79 fragment (residues 1–75) inhib-

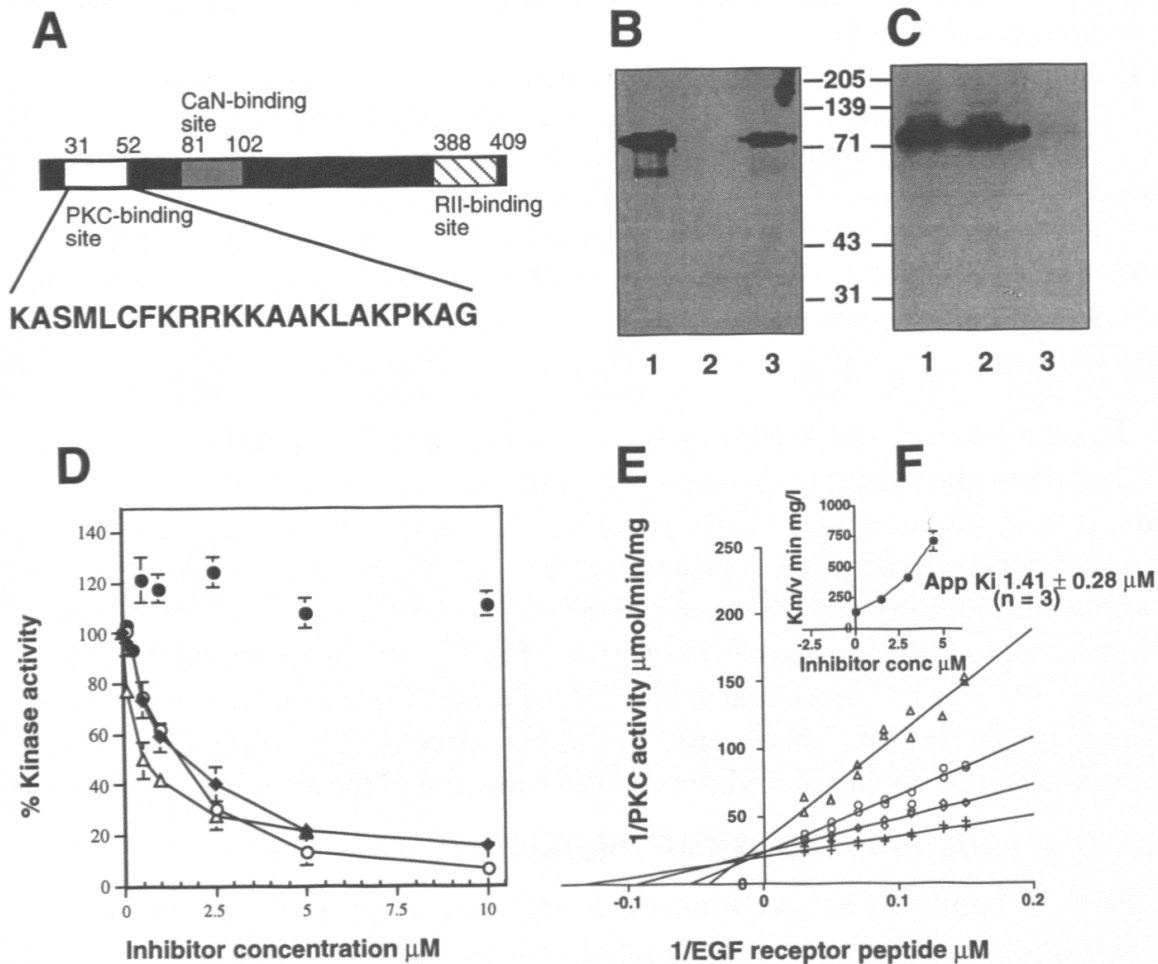


Fig. 7. Mapping the PKC-binding site on AKAP79. (A) Schematic diagram of AKAP79 showing binding sites for PKA, CaN, and PKC. The amino acid sequence for residues 31–52 is indicated. (B) Recombinant AKAP79 was blotted and PKC overlays were performed in the absence (lane 1) and presence of either 1.5 μM AKAP79(31–52) (lane 2) or 1.5 μM RII-anchoring inhibitor peptide AKAP79(390–412) (lane 3) with ~ 12.5 nM PKC. (C) ^{32}P -RII overlays were performed under the same conditions as in B. (D) Dose–response curve of PKC activity in the presence of recombinant AKAP79 (open triangles), AKAP79(31–52) (closed diamonds), and residues 1–75 recombinant fragment of AKAP79 (open circles). AKAP79(31–52) did not inhibit PKA activity (closed circles). (E) Lineweaver–Burk plot of PKC phosphorylation in the absence of inhibitor peptide (crosses) and in the presence of 1.5 μM (open diamonds), 3 μM (open circles) and 4.5 μM (open triangles) AKAP79 (31–52) peptide. Inset (F) shows the secondary plot of K_m/V_{max} as a function of AKAP79(31–52) concentration and the apparent K_i value. Values given are as mean \pm SEM (15).

ited PKC ($\text{IC}_{50} = 2.0 \pm 0.6 \mu\text{M}$, $n = 4$) and ($\text{IC}_{50} = 1.6 \pm 0.3 \mu\text{M}$, $n = 4$) (Fig. 7D [18]; see Subheading 3.3.2.). In contrast, the 31–52 peptide did not inhibit the activity of the catalytic subunit of PKA (Fig. 7D; ref. 18). Inhibition of PKC

activity by the 31–52 peptide was mixed with an apparent inhibition constant (K_i) of $1.41 \pm 0.28 \mu\text{M}$ ($n = 3$) (**Fig. 7E**). The secondary plot of the Michaelis constant divided by the maximal velocity (K_m/V_{max}) as a function of inhibitor concentration, was nonlinear, suggesting binding at more than one site (**Fig. 7F**). These peptide studies were powerful, in that they allowed us to map a site of contact to residues 31–52 on the AKAP.

3.3.1. Peptide Block of PKC-AKAP Interaction in PKC Overlay

The PKC overlay is similar to the RII overlay (*see Subheading 3.1.1.*) except that an antibody to PKC is used to detect the PKC bound to PKC-binding proteins (**24**).

1. Separate protein samples by SDS-PAGE and transfer to nitrocellulose membrane by standard electrotransfer techniques (**ref. 33**; *see Note 1*).
2. Wash 3×5 min with TBS and block membrane by incubation with BLOTTO + 1% BSA for 30 min at room temperature (*see Note 2*).
3. Wash 3×5 min with TBS.
4. Incubate blot for 1 h with PKC (approx $10 \mu\text{g/mL}$, but this should be titrated for each prep) \pm anchoring peptides ($1.5 \mu\text{M}$) and $20 \mu\text{g/mL}$ phosphatidylserine (PS) or 1.4 mM PS/ 0.04 mM diacylglycerol (DG) vesicles (*see Notes 23 and 26*) in PKC assay buffer (TBS, 1% albumin, 1.2 mM Ca^{2+} , 1 mM EGTA, and protease inhibitors [leupeptin and pepstatin $\{2 \mu\text{g/mL}\}$]).
5. Wash blot with PBS wash buffer (PBS containing 1.2 mM Ca^{2+} , 1 mM EGTA, $10 \mu\text{g/mL}$ PS) for 2×3 min to remove excess PKC.
6. Fix blot with 0.5% paraformaldehyde in PBS wash buffer for 20 min at room temperature (*see Note 27*) (optional).
7. Incubate with PBS + 2% glycine, pH 7.4, for 20 min at room temperature to block free amine groups.
8. Wash 2×5 -min with TBS.
9. Develop as for standard Western blot with primary antibody to PKC to detect PKC-binding proteins. Develop by chemiluminescence.

3.3.2. Peptide Inhibition of PKC Activity

3.3.2.1. PKC ACTIVITY ASSAYS

Protein kinase assay using phosphocellulose cation exchange paper (P81 paper). This method relies on the basic nature of the substrate. Because many kinases have requirements for basic residues, and synthetic peptides have proved to be good substrates, this method is ideal for assaying kinase activity. There are many peptide substrates available commercially. We have routinely used a peptide to the EGF-receptor (VRKRTLRL) available from Sigma.

1. Make stocks containing 4X concentrate assay buffer, 10X CaCl_2 , 10X EGTA, and 10X PS/DG vesicles and the $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ stock (200 cpm/pmol; 1 mM [*see Section 3.2.1.1.*]). The final concentration in the assay should be: 20 mM HEPES,

pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.3 mM Ca²⁺ (or 0.5 mM EGTA), 0.14 mM PS/0.004 mM DG in 2 mM HEPES, pH 7.5 (*see Note 23*) (or 2 mM HEPES, pH 7.5), and 0.1 mM [γ -³²P]-ATP.

2. In a 40- μ L reaction volume, add the appropriate volume of concentrated stocks (*see step 1*) with peptide substrate, vortex, and preincubate at 30°C.
3. Dilute PKC in ice-cold buffer (20 mM Tris, pH 7.9, containing 1 mg/mL BSA, 1 mM DTT) and start reaction by addition of diluted enzyme at 20-s intervals, vortex, and incubate at 30°C (*see Note 24*).
4. The reaction is stopped by taking 30- μ L aliquots from each tube at 20-s intervals, spotting on to 2 \times 2 cm P81 paper, and dropping into 75 mM phosphoric acid (*see Note 25*).
5. When all time-points have been taken, pour the first wash into radioactive waste and wash (2 \times 3 min) with 500 mL 75 mM phosphoric acid. Wash once with ethanol and dry papers before counting by liquid scintillation.

3.3.2.2. INHIBITION OF PKC

To determine the half-maximal inhibitor constant, assay PKC as described above, in the presence of increasing amounts of inhibitor peptide. To assay for inhibition, it is important to assay enzyme activity at K_m for the substrate peptide. The K_m can be determined by assaying PKC over a range of substrate concentrations (as described in **Subheading 3.2.2.**). The half-maximal inhibition of the peptide inhibitor is determined by plotting the percentage of PKC activity vs inhibitor concentration. To determine the K_i and the mechanism of inhibition, the enzyme is assayed over a range of substrate concentrations at varying inhibitor concentrations (*see Fig. 7E,F*). The Lineweaver-Burk plots for each inhibitor concentration are plotted on the same axis. If inhibition is competitive with respect to peptide substrate, the Lineweaver-Burk plots intersect at the Y-axis at $1/V_{max}$. If inhibition is noncompetitive, they intersect at the X-axis at $1/K_m$. The K_i value is then determined from a secondary plot of K_m/V_{max} versus inhibitor concentration where the inhibitory constant intersects at the X-axis at $-K_i$.

3.4. Conclusions

The studies outlined in this chapter have highlighted the use of synthetic peptides as tools to evaluate the interactions between anchoring proteins and signaling enzymes. In particular, peptides have been used to map biologically important binding sites on specific AKAPs. The advantages of peptides over recombinant proteins is that a relatively short sequence (for example, 20 amino acids) can be custom-synthesized in large quantities and can be quickly used to define biologically important sites. Stoichiometric incorporation of myristoyl or phosphate moieties into the polypeptide often significantly enhances the action of certain peptides. For example, the esterification of aliphatic lipids

can significantly enhance the versatility of peptide reagents by rendering them cell-soluble. No doubt the development of additional cell-soluble peptides for use in cell-based assays will be a topic of considerable interest to both researchers and commercial entities alike.

Although the generation of cell-soluble peptides increases the versatility of certain reagents, a major concern remains their susceptibility to proteolysis *in vivo*. For example, the radioiodinated PKA-inhibitor peptide, PKI 5-24, has a half-life of a few seconds when microinjected into *Xenopus* oocytes (M. F. Cicerelli and J. D. Scott, unpublished observation). Nevertheless, numerous studies have demonstrated that the PKI 5-24 peptide is very effective in broken cell extracts or when introduced at high concentrations. Another limitation of peptide fragments is that they often exhibit a diminished biological potency, compared to their parent protein. For example, the PKI 5-24 peptide has a K_i of 4–8 nM, and the parent protein inhibits the C subunit with a K_i of 0.23 nM (29). Likewise, the anchoring-inhibitor peptide Ht 31 493–515 binds RII or the type II PKA holoenzyme with a K_d of 3.8–4 nM; however, it is clear that a recombinant fragment of the Ht 31 protein binds with much higher affinity. Although bioactive peptides, such as PKI 5-24 and Ht 31 495–515, which act in the nanomolar range, may be suitable for certain cell based assays, it is less clear whether peptides that are active in the micromolar range will be active in cells. Despite these limitations, it is clear that synthetic peptides will continue to be valuable reagents in the dissecting protein-targeting interactions.

4. Notes

1. The transferred proteins on the membrane can be visualized by staining with Coomassie blue (46% methanol/9% acetic acid/0.05% Coomassie) for Immobilon or Ponceau stain (0.2% Ponceau in 1% acetic acid) for nitrocellulose. Stain for 1–2 min and then destain with 46% methanol/9% acetic acid for Immobilon, or with water for the nitrocellulose. This is very useful to correlate nonspecific binding to abundant proteins in an extract.
2. Blots are often incubated overnight.
3. Collect 200- μ L fractions from column and count 1 μ L by Cerenkov counting. Expect the radiolabeled protein to elute in fraction 4 or 5 with 100,000 cpm/ μ L.
4. It is important to incubate an identical blot with control peptide, or in the absence of peptide, to be sure that the RII-binding protein is present.
5. Blots are often incubated overnight.
6. This can be done at room temperature. This step is important for removing non-specific proteins.
7. Either the sodium salt of cAMP or the free acid (SIGMA) can be used, but to use the free acid, it is necessary to adjust the pH of the solution.
8. It is very important that incubation occurs at room temperature for both the peptide elution and the cAMP elution.

9. The amounts of protein present in the elution are often very small, and so it is important to load all of the sample.
10. It was necessary to dilute the anchoring inhibitor peptide in DMSO, because it is very hydrophobic and not very soluble.
11. Rat hippocampal neurons were prepared from neonatal rats and cultured on rat astrocyte cultures for 2 wk.
12. For the myristoylated Ht31 peptide (493–509), an affinity-purified antibody to the peptide was used at a 1:200 dilution.
13. $1 \mu\text{Ci} = 2.2 \times 10^6 \text{ cpm}$. Decay tables are available to calculate the radioactive concentration and specific activity of the stock. When making the cold ATP stock, it is important to neutralize the solution to pH 7.0. This stock can be aliquoted and frozen at -20°C .
14. Take four $3\text{-}\mu\text{L}$ aliquots to count and spot on P81 paper. Wash two aliquots with 75 mM phosphoric acid ($3 \times 3 \text{ min}$ washes), wash with ethanol ($1 \times 2 \text{ min}$), and dry. Count Cerenkov radiation for both washed ^{32}P -RII and unwashed (total counts).
15. AG1X8 resin is an anion exchange resin used to separate free $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ from radiolabeled peptide. We pour the resin in a glass Pasteur pipet with a glass bead at the base.
16. Activate C-18 cartridge by washing with $100\% \text{ CH}_3 \text{ CN}$, $0.1\% \text{ TFA}$ (10 mL), followed by washing with $0.1\% \text{ TFA}$ (10 mL).
17. It is important to collect 1-mL aliquots of eluted ^{32}P -RII because $>90\%$ should elute in the first mL.
18. To calculate incorporation:

$$1 \text{ mM RII}/10^{-3} \text{ mol/L} \times 4 \times 10^{-6} \text{ L} = 400 \times 10^3 \text{ pmol}$$

$$400 \times 10^3 \text{ pmol}/520 \mu\text{L} = \text{pmol}/\mu\text{L} \times 10 \mu\text{L} = \text{pmol RII}$$

(reaction volume $400 \mu\text{L}$, final vol $520 \mu\text{L}$)

$$\text{pmol RII} \times \text{cpm}/\text{pmol} [\gamma\text{-}^{32}\text{P}]\text{-ATP} = \text{cpm expected for 1:1 incorporation}$$

19. To calculate concentration of ^{32}P -RII:

$$\text{cpm (of final } 2\text{-}\mu\text{L aliquot)}/\text{cpm}/\text{pmol} [\gamma\text{-}^{32}\text{P}]\text{-ATP} \times 2 = \text{pmol}/\mu\text{L}$$

20. Always include a background control, containing no enzyme.
21. The reaction should be carried out so that the rate of dephosphorylation is linear and less than 10% of the substrate is dephosphorylated. This will mean that the reaction time will vary, but is generally between 5 and 10 min .
22. We have used either glass Pasteur pipets with a glass bead placed at the bottom or BIORAD polyprep chromatography columns.
23. To prepare PS/DG vesicles: PS and DG are stored at -20°C in chloroform (Avanti Polar Lipids), and must be aliquoted using glass pipet tips or a Hamilton syringe, and stored in glass tubes. Aliquot the appropriate volume of PS and DG and dry under a constant stream of nitrogen gas. Resuspend in 20 mM HEPES, pH 7.5 , sonicate for 30 s , and store at 4°C . It is a good idea to make fresh solutions every couple of weeks. The solution will be opaque, even following sonication.

24. For inhibition assays, enzyme can be preincubated with inhibitor and reaction started with reaction mix containing buffer, activators, and substrates, or the inhibitor can be added to the reaction mix and reaction initiated with enzyme.
25. A convenient way to do this is to place the spotted P81 papers into a wire cage inside a 500-mL beaker containing 75 mM phosphoric acid. The beaker is placed on a magnetic stir plate. The papers are kept in the wire cage until they are dried prior to counting.
26. PS/DG vesicles are sometimes used in preference to PS.
27. Make from 3.7% paraformaldehyde PBS stock.

Acknowledgments

The authors wish to thank their colleagues at the Vollum Institute for critical evaluation of this manuscript and Ruth Frank for her assistance in the preparation of this manuscript. M. F. and J. D. S. were supported in part by DK 44239.

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