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Overlay, Ligand Blotting, and Band-Shift Techniques to Study Kinase Anchoring

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1. Introduction

One common theme in protein–protein interactions is that binding often occurs through relatively small domains. This is particularly true in the area of signal transduction, in which numerous studies have demonstrated that the subcellular location of protein kinases and phosphatases is regulated through association with structural proteins or cellular organelles *(1,2)*. This has led to the proposal by Hubbard and Cohen of a targeting hypothesis, in which both classes of enzyme are maintained in close proximity to specific substrates by targeting domains *(3)*. Kinase and phosphatase targeting not only facilitates the rapid and preferential phosphorylation of substrates, but optimally positions these enzymes at sites where they can be efficiently activated in response to the appropriate signals *(3)*.

Evidence supporting this model has shown that protein tyrosine kinases and phosphatases couple to downstream cytoplasmic enzymes through modular adapter proteins that contain Src homology (SH2 and SH3) domains *(4)*. Both of these peptide-binding domains are conserved secondary structures that specifically recognize linear sequence motifs on their recipient proteins *(5,6)*. Serine and threonine kinases and phosphatases use variations on this theme to achieve their correct subcellular targeting *(2)*. Phosphatases type I and 2A (PP-1 and PP-2A) are localized by a growing family of targeting subunits *(7,8)*, and phosphatase 2B, calcineurin, is targeted to the postsynaptic densities of neurons *(9)*. Compartmentalization of protein kinase C (PKC) occurs through interaction with substrate-binding proteins, sometimes called receptors for
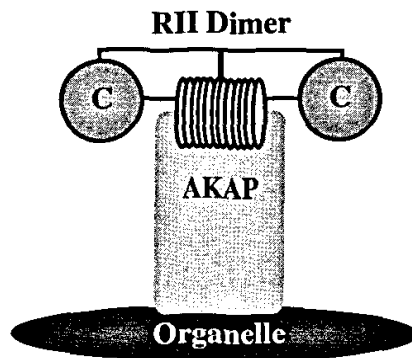
Fig. 1. Models for RII–AKAP interaction. A schematic diagram depicting possible topologies of RII–AKAP interaction.

activated c-kinases (RACKs) *(10,11)*. Subcellular targeting of the cAMP-dependent protein kinase (PKA) occurs through its association with A-kinase anchoring proteins (AKAPs) *(12,13)*. Work on PKA anchoring has been facilitated by the development of several in vitro binding techniques, such as the solid-phase overlay, interaction cloning strategies and band-shift analysis, which exploit the properties of PKA–AKAP interaction *(14)*. The goal of this chapter is to use PKA anchoring as a model system to describe these techniques and outline their use for investigating protein/protein interactions. Special emphasis has been placed on providing the reader with the precise protocols, presented in a step by step approach, that highlight the advantages and pitfalls of each procedure.

1.1. PKA–AKAP Interaction

PKA anchoring is directed through the association of the regulatory (R) subunit dimer with an amphipathic helix on the anchoring protein (**Fig. 1**). Although there have been a few isolated reports of PKA anchoring directed through the type I R subunit (RI) *(15)*, it is generally accepted that the type II R subunit (RII) is the principle targeting subunit *(16,17)*. Mapping of the AKAP-binding site on RII has demonstrated that dimerization is required for anchoring *(18)*. Deletion analysis has suggested that the localization and dimerization domains are contained within distinct but overlapping regions within the first 30 residues of each RII protomer *(19)*. In addition, mutagenesis studies have identified two isoleucine side chains on each RII protomer as principle sites of contact with the anchoring protein (**Fig. 1**), but other studies have demonstrated that additional hydrophobic contacts occur in regions downstream of these isoleucines *(19,20)*.

In contrast, the RII-binding site on the AKAP seems to involve a region of secondary structure that is confined to a short stretch of amino acids. Deletion

analysis and mutagenesis studies have demonstrated regions of 14–24 residues on several AKAPs that exhibit a high probability of amphipathic α-helix formation are essential for anchoring *(21–24)*. In fact, peptides corresponding to these helical regions specifically bind RII or the type II PKA holoenzyme with a high affinity (K_d ~4 n*M*) *(25)*. Recently, these peptides have been used as potent anchoring inhibitors to disrupt the localization of PKA in vivo *(26,27)*. The AKAPs' compact binding domain makes RII–AKAP interactions particularly amenable to analysis by the binding techniques described in this chapter. The following sections provide detailed protocols for in vitro binding techniques, such as solid-phase overlay assays (*see* **Subheading 3.1.**), overlay-based cloning strategies (*see* **Subheading 3.2.**), peptide overlays (*see* **Subheading 3.3.**), and band-shift analysis (*see* **Subheading 3.4.**).

2. Materials

2.1. Protein Overlay Techniques

2.1.1. Radioactive (^{32}P) RII Overlay

1. Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE).
2. Immobilon or nitrocellulose.
3. Immobilon stain: 46% Methanol, 9% acetic acid, 0.05% Coomassie blue; or Ponceau stain: 0.2% Ponceau in 1% acetic acid.
4. Immobilon destain: 46% methanol, 9% acetic acid.
5. BLOTTO: Tris-buffered saline, pH 7.0 (TBS), 5% dry milk, 1% bovine serum albumin (BSA).
6. RII (2 µg).
7. PKA catalytic subunit (0.1 µg) and [γ-^{32}P] ATP (50 µCi).
8. PKA reaction buffer: 50 m*M* MOPS, pH 6.8, 50 m*M* NaCl, 2 m*M* MgCl$_2$, 1 m*M* DTT, 0.1 mg/mL BSA.
9. TBS/0.05% Tween-20 (TTBS).
10. 3MM paper.
11. 2X sample buffer: 100 m*M* DTT, 2% SDS, 80 m*M* Tris-HCl, pH 6.8, 10% glycerol, and 0.0012% bromophenol blue.
12. Vertical electrophoresis apparatus and gel solutions.
13. Gel transfer apparatus.

2.1.2. Nonradioactive RII Overlay

1. 0.5 µg/mL of BLOTTO.
2. TTBS.
3. Anti-RII antibodies (1:20,000).
4. HRP-conjugated secondary antibody (1:10,000).
5. Chemiluminescence kit.
6. SDS-PAGE.
7. Immobilon or nitrocellulose.

8. Immobilon stain: 46% Methanol, 9% acetic acid, 0.05% Coomassie blue, or Ponceau stain: 0.2% Ponceau in 1% acetic acid.
9. Immobilon destain: 46% methanol, 9% acetic acid.
10. RII (2 μg).
11. 3MM paper.
12. 2X sample buffer: 100 mM DTT, 2% SDS, 80 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.0012% bromophenol blue.
13. Vertical electrophoresis apparatus and gel solutions.
14. Gel transfer apparatus.

2.1.3. Slot-Blot Overlay

1. Nitrocellulose filter.
2. Target protein ~2 mg.
3. Slot-blot manifold.
4. BLOTTO: TBS, pH 7.0, 5% dry milk, 1% BSA.
5. Bait protein (^{32}P-labeled or antibait protein antibodies).

2.2. Interaction Screening

2.2.1. Radioactive RII Interaction Screening

1. PKA catalytic subunit (0.1 μg) and γ^{32}P ATP (50 μCi).
2. PKA reaction buffer: 50 mM MOPs, pH 6.8, 50 mM NaCl, 2 mM MgCl$_2$, 1 mM DTT, 0.1 mg/mL BSA.
3. Bacteriophage λ (e.g., λgt11 or λZap) cDNA expression library.
4. IPTG-soaked nitrocellulose filters.
5. BLOTTO: TBS, pH 7.0, 5% dry milk, 1% BSA.
6. Bait protein.
7. TBS/0.05% Tween-20 (TTBS).
8. Plastic wrap.
9. Autoradiography film.

2.2.2. Nonradioactive RII Interaction Screening

1. Bacteriophage λ (e.g. λgt11 or λZap) cDNA expression library.
2. BLOTTO: TBS, pH 7.0, 5% dry milk, 1% BSA.
3. Recombinant RII.
4. TBS.
5. Primary antibody (1:5000).
6. HRP-linked secondary antibody.
7. Chemiluminescent detection reagents.
8. Autoradiography film.

2.3. Peptide Overlay

1. Nitrocellulose filter.
2. TBS.
3. Slot-blot manifold.

4. RII proteins.
5. BLOTTO: TBS, pH 7.0, 5% dry milk, 1% BSA.
6. Biotinylated RII-binding peptide (0.4 mM).
7. TTBS.
8. 1:10,000 dilution of streptavidin-HRP.

2.4. Band-Shift Assay

1. Nondenaturing (ND) PAGE gels.
2. Binding buffer: 5 mM HEPES, 10 mM DTT, 1 mM benzamidine, 100 mM KCl, and 0.01% Tween.
3. ND sample buffer: 100 mM MOPS, 40% glycerol, 0.01% bromophenol blue.
4. Coomassie based stain.
5. Fresh acrylamide stock.
6. Fresh binding buffer.
7. Isobutanol.
8. Vertical electrophoresis apparatus.
9. Gel solutions (omitting SDS).

3. Methods

3.1. Protein Overlay Techniques

3.1.1. The RII-Overlay

Detailed study of PKA anchoring was made possible by the original observation of Lohmann and colleagues *(16)* that many, though most likely not all, AKAPs retain their ability to bind RII after transfer to nitrocellulose. As a result, an overlay technique has been developed that is essentially a modification of the Western blot procedure, in which the typical antibody probe is replaced by ^{32}P labeled RII protein *(14)*. The overlay technique (*see* **Subheading 3.1.1.1.**) is summarized in **Fig. 2**. Using this approach, we have been able to survey AKAPs from a variety of tissue sources (**Fig. 3A**). A routine control experiment that determines specificity uses an anchoring inhibitor peptide to block binding (**Fig. 3B**). For greater precision the basic overlay technique can be used after a two dimensional separation of proteins (**Fig. 3C**). Samples are initially separated by isoelectric focusing (IEF) in the first dimension, and by SDS-PAGE in the second dimension. After electrotransfer, the standard overlay procedure is performed. Another adaptation is the development of nonradioactive methods for detection of RII binding (*see* **Subheading 3.1.1.2.**). The use of chemiluminescence to detect RII–AKAP complexes increases the sensitivity of this technique 20- to 50-fold.

3.1.1.1. RADIOACTIVE (^{32}P) RII OVERLAY

1. Protein samples are separated by SDS-PAGE and transferred to immobilon or nitrocellulose by standard electrotransfer techniques (**ref. *28*;** *see* **Note 1**).
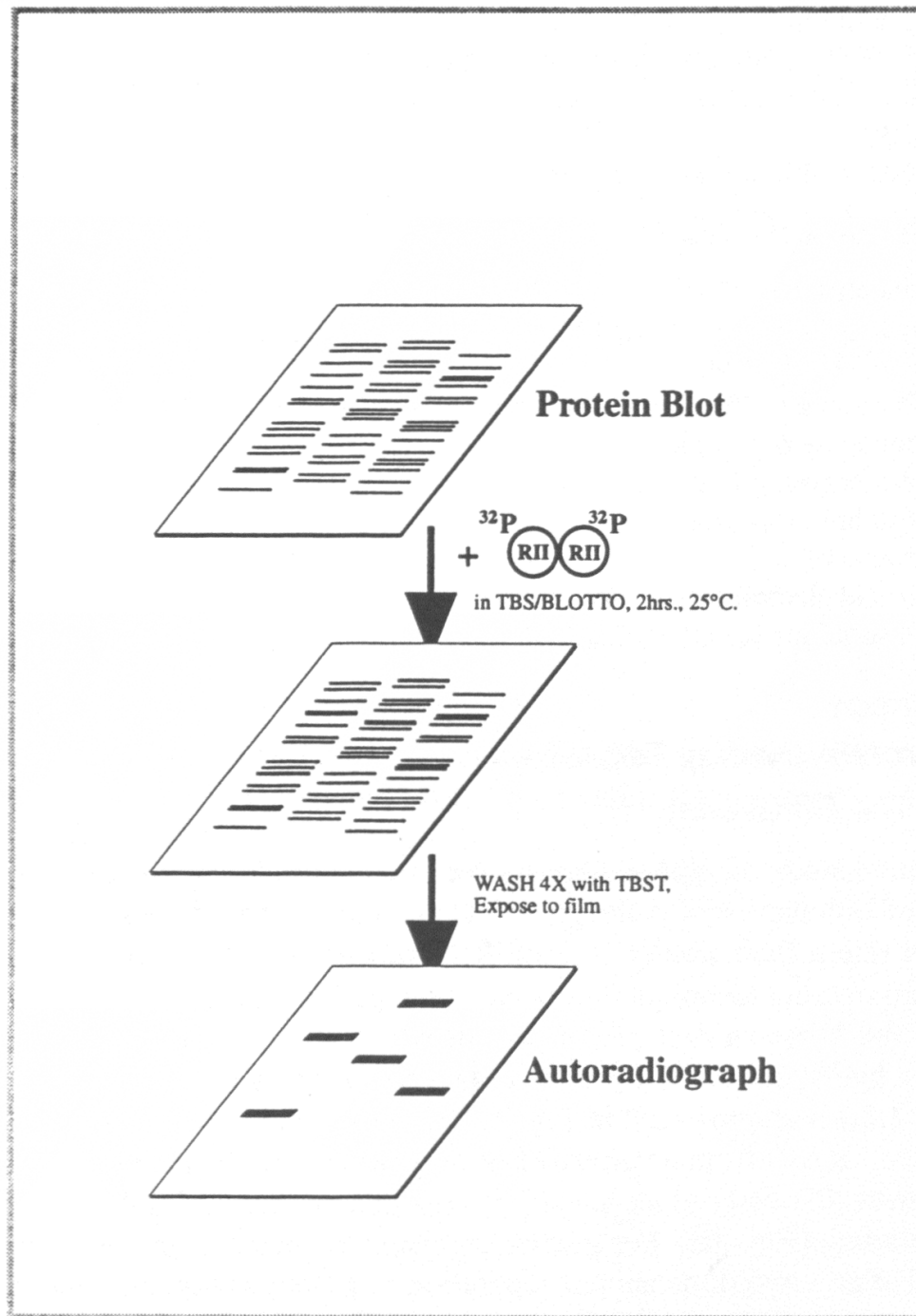
Fig. 2. The RII overlay. A schematic diagram of the RII overlay.

2. Stain bound proteins with Immobilon stain or Ponceau stain for nitrocellulose (*see* **Subheading 2.1.1., item 3**).

3. Destain the filter with Immobilon destain (*see* **Subheading 2.1.1., item 4**) or with water for the nitrocellulose (*see* **Note 2**).

4. The membrane is blocked by incubation in BLOTTO + BSA (TBS, pH 7.0), 5% dry milk, 1% BSA for 1 h.
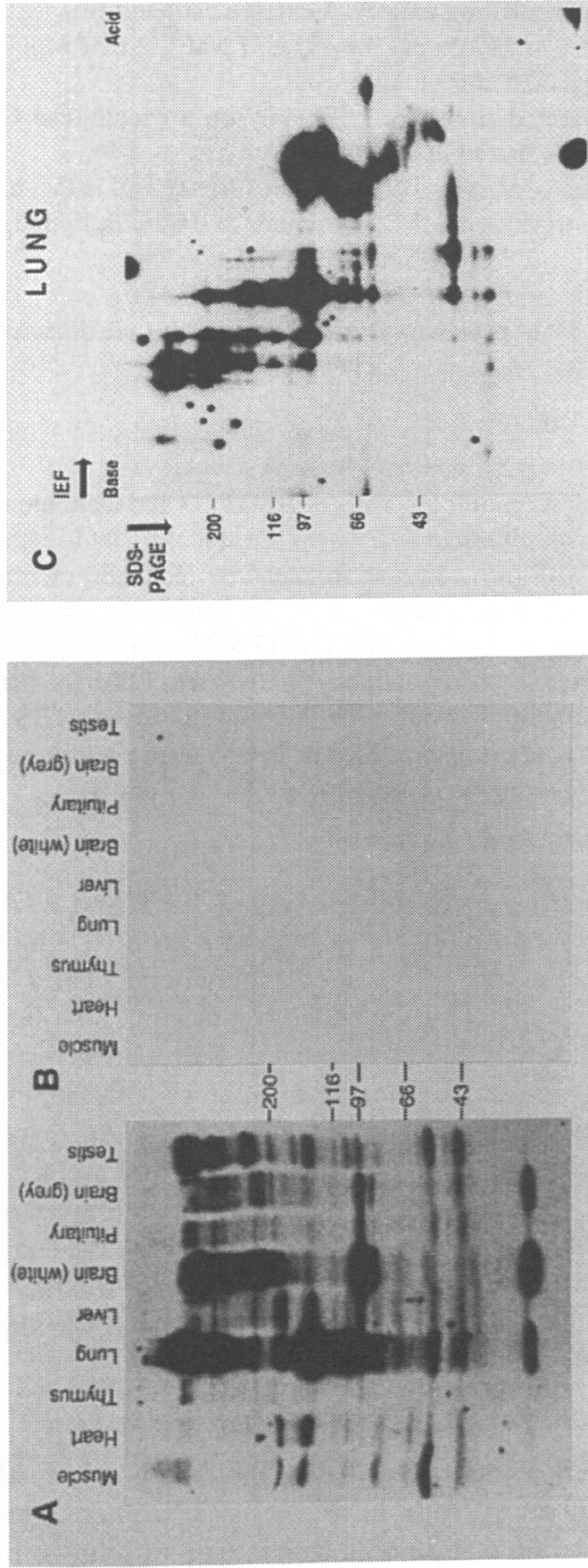
Fig. 3. Applications of the overlay. Crude protein extracts from nine bovine tissues were analyzed by RII overlay for anchoring proteins. Protein samples (50 μg) were separated by electrophoresis on a 4–12% SDS polyacrylamide gradient gel. After electrotransfer to immobilon, RII-binding proteins were detected, as described in the experimental methods. Two identical blots were incubated with either ^{32}P RIIα (**A**) or ^{32}P RIIa in the presence of 0.4 μM Ht 31 (493–515) peptide (**B**). Tissue sources are marked above each lane (Reproduced with permission from *J. Biol. Chem.*). (**C**) Protein extract from bovine lung was separated by isoelectric focusing in the first dimension and electrophoresis on 4–12% gradient SDS-PAGE gels before transfer to Immobilon. RII-anchoring proteins were detected by autoradiography after incubation of the blot with ^{32}P RIIα. The direction of IEF and molwt markers are indicated on the sides of each overlay. (Reproduced with permission of *Trends Biochem. Sci.*)

5. RII (2 µg) is radiolabeled by incubation with PKA catalytic subunit (0.1 µg) and [γ-^{32}P]-ATP (50 µCi), in 50 mM MOPs, pH 6.8, 50 mM NaCl, 2 mM MgCl$_2$, 1 mM DTT, 0.1 mg/mL BSA at 30°C for 15 min.

6. Radiolabeled protein is separated from free [^{32}P] ATP on a excellulose GF-5 desalting column (Pierce) equilibrated in TTBS.

7. Radiolabeled RII probe (specific activity 10^5 cpm/mL of BLOTTO) is incubated with the blocked membrane from step 4 for a minimum of 3 h, with light agitation. (*see* **Note 3**).

8. Wash with TTBS (3 × 15 min) to remove free ^{32}P RII.

9. Filter is dried by blotting on 3MM paper and RII-binding proteins are detected by autoradiography (*see* **Note 4**).

3.1.1.2. Nonradioactive RII Overlay

1. Follow **steps 1–4** of **Subheading 3.1.1.1.** (*see* **Note 5**).

2. Add RII protein to a concentration of 0.5 µg/mL of BLOTTO and incubate with the blocked membrane for a minimum of 3 h.

3. The filter is washed with TTBS (3 × 15 min), followed by incubation with RII antibodies (*see* **Note 6**).

4. Excess antibody is removed by washing with TTBS (3 × 15 min), followed by incubation with HRP-conjugated secondary antibody (1:10,000) in TTBS for 40 min.

5. Uncomplexed secondary antibody is removed by washing in excess TTBS (3 × 15 min), and the immune complex is detected using an enhanced chemiluminescence kit (Renaissance by New England Nuclear) (*see* **Notes 7 and 8**).

3.1.2. AKAP Overlay

The AKAP overlay is a modification of **Subheadings 3.1.1.1.** and **3.1.1.2.**, except that RII is immobilized and the AKAP is used as a probe. Using this technique, RII–AKAP complexes can be detected immunochemically with anti-AKAP antibodies or by radiolabeling the anchoring protein. One limitation of this technique seems to be that small fragments of RII, such as residues 1–50, which bind AKAPs in solution, are unable to interact with anchoring proteins when immobilized to the solid-phase support *(19,20)*. An apparent explanation for this observation is that a larger percentage of the total surface area on small proteins is required for contact with the solid-phase membrane than in larger protein. Accordingly, this may decrease the number of active binding sites that are accessible to the AKAP. Nevertheless, the AKAP overlay is a simple technique that can be used to rapidly screen RII mutants for altered AKAP-binding properties *(20)*.

3.1.3. Semiquantitative Overlays (Slot-Blot Analysis)

A valid criticism of the overlay procedures is that they are purely qualitative. Therefore, we have made an effort to adapt the basic technique into a semiquantitative overlay procedure that permits comparison of relative bind-
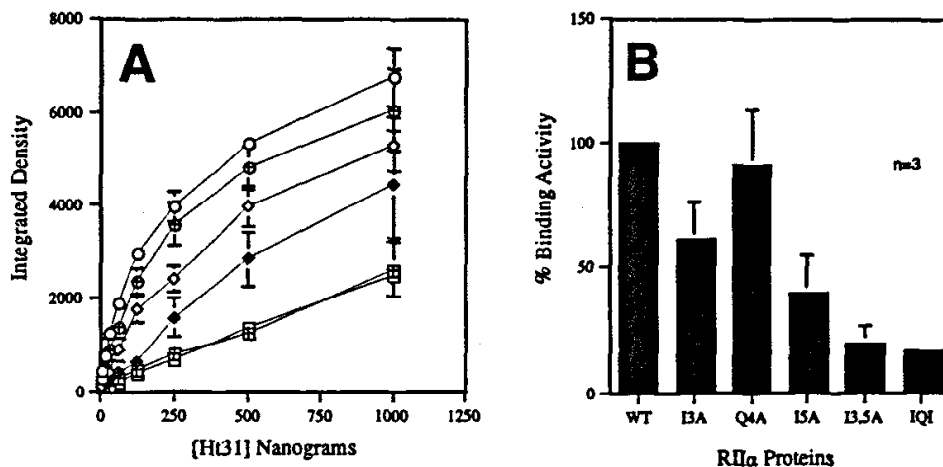
Fig. 4. Slot-blot overlay. Quantitation of AKAP binding to RIIα mutants. The binding of RIIα and mutants to a recombinant fragment of the human thyroid anchoring protein Ht31 was measured by a quantitative overlay procedure. **(A)** Binding curves for individual protein probes from three experiments are RIIα (o), RIIα I3A (◊), RIIα Q4A (⊕), RIIα I5A (♦), RIIα I3A I5A (□) and RIIα IQI, AAA (⊞). **(B)** The degree of Ht31 binding obtained at a single concentration of protein (25 ng) is presented as a percent binding compared with wild-type RIIα. Data are presented with permission from the *J. Biol. Chem.*

ing affinities for various RII–AKAP interactions. This was achieved by using a slot-blot manifold to immobilize the proteins on the surface of the membrane in a uniform manner. Accordingly, a filter of immobilized proteins (over a range of concentrations) is probed with a ligand of known specific activity. The linear range of the assay can be determined by monitoring the intensity of signal at each concentration of immobilized protein. This information is necessary for comparison of various binding affinities. One advantage of the slot-blot overlay is that a single filter can be used to compare the binding affinity of several RIIα mutants for an individual AKAP *(19)*. Alternatively, a single RII mutant can be used to screen for altered binding properties with a variety of AKAPs **(Fig. 4)**.

3.1.3.1. SLOT-BLOT OVERLAY

1. Soak a nitrocellulose filter with 0.2 mL of TBS using a slot-blot manifold (e.g., Hybri-slot, BRL) (*see* **Note 9**).
2. Target proteins (RII or AKAP) are immobilized onto nitrocellulose at various concentration over a range of 0.3 ng–100 ng, using the slot-blot manifold (*see* **Note 10**).
3. The membrane is blocked in BLOTTO for 1 h.
4. Probe membrane with bait protein. If using a radioactive protein probe continue following **steps 5–9** of **Subheading 3.1.1.1.** For a nonradioactive protein probe, continue following **steps 2–5** of **Subheading 3.1.1.2.** (*see* **Note 11**).

5. The relative intensity of binding is measured by densitometric analysis of the autoradiogram. This is achieved by digitally scanning images into a computer and analyzing the band density using the NIH image program (*see* **Note 12**).

6. Numeric values correlating to band intensity can now be plotted against concentration to obtain binding curves (*see* **Note 13**).

3.1.4. Alternate Detection Methods

The detection methods described above are useful if antibodies to the protein probe are available or if the protein probe can be radiolabeled. For example, RII is an excellent probe because it has a autophosphorylation site that allows incorporation of stoichiometric levels of ^{32}P into the protein. However, a number of alternative methods are available to detect the protein probe. One vector developed in our laboratory, called pET-kfc, produces chimeric recombinant proteins in *Escherichia coli* containing a phosphorylatable affinity ligand *(29)*. The kfc tail is a 51 residue peptide that encodes a consensus PKA phosphorylation site LRRA**S**LG (**K**), a restriction proteinase site for Factor X (**F**), and a high-affinity calmodulin binding domain (**C**). Recombinant kfc-proteins can be purified in one step by affinity chromatography on calmodulin-Sepharose, and are phosphorylated to a stoichiometry of 1 by the C subunit of PKA. Furthermore, immobilized kfc proteins can be detected by a solid-phase overlay technique, using biotinylated calmodulin as a probe *(29)*. Other affinity fusion tags are commercially available, such as glutathione-*S*-transferase (GST) and His T7 tag, which permit rapid one-step purification of recombinant proteins *(30,31)*. Again, antibodies are available to both of these affinity tags, permitting detection of the immobilized fusion protein in overlay assays.

3.2. Overlay Cloning Strategies

The *in situ* overlay method is easily adapted to screening cDNA expression libraries for proteins that associate with the ligand or bait protein (e.g., RII). The technique is quite sensitive, since bacterial expressed products often adopt their native conformation. Two methods for overlay screening are described. The first method is a direct adaptation of the *in situ* overlay described in **Subheading 3.1.** using radiolabeled bait protein as a probe. Because radiolabeling is not always possible, or may interfere with binding, a second method of screening is described that uses unlabeled bait protein and antibody detection (similar to **Subheading 3.1.2.**) (**Fig. 5**).

3.2.1. Radioactive RII Interaction Screening

1. Plate bacteriophage λ (e.g., λgt11 or λZap) cDNA expression library at 1–5 × 10⁴ PFU/150-mm plate and perform lifts with IPTG-soaked nitrocellulose filters, as described by Sambrook et al. *(32)*. Save master plates at 4°C.
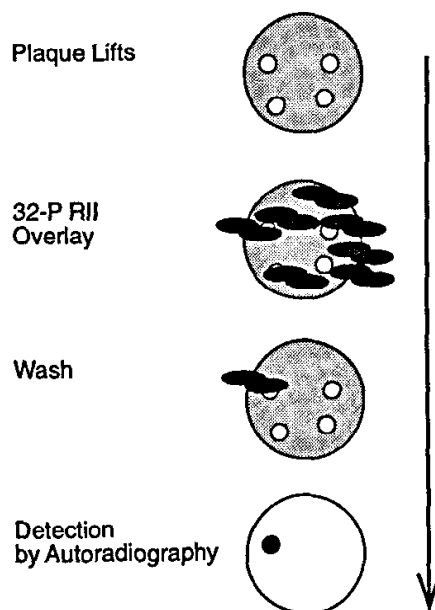
Fig. 5. An overlay expression cloning strategy. A schematic diagram of the an interaction cloning strategy that uses radioactive RII as a probe.

2. Block filters in BLOTTO for 2 h at room temperature.
3. Radiolabel bait protein, as described in **Subheading 3.1.1.1. (steps 5 and 6)** and incubate with filters at ~5 × 10⁴ cpm/mL of BLOTTO or other binding media for 4–16 h at room temperature.
4. Decant incubation solution into radioactive liquid waste and wash filters in BLOTTO or TBST for 15 min. Repeat washes three times.
5. Blot dry filters, cover with plastic wrap, and expose to autoradiography film for 1–3 d.
6. Align film to master plates. Pick positive plaques, replate, and perform secondary screenings using **steps 2–5**.

3.2.2. Nonradioactive RII Interaction Screening

1. Follow **steps 1–2 in Subheading 3.2.1.**
2. Incubate blocked filters with purified bait protein in BLOTTO or other binding buffer for 4 h at room temperature. For cloning of RII binding proteins, we use 0.5 µg of recombinant RII per mL of BLOTTO.
3. Wash filters with TBS for 15 min. Repeat washes three times.
4. Incubate the filters with primary antibody in TBS for 2 h at room temperature. We use our antibodies at dilutions similar to those used in Western blots (1:5000 for affinity-purified antibody against RII).
5. Wash filters as in **step 3**.
6. Incubate the filters with HRP-linked secondary antibody in TBS for 1 h at room temperature. Use the same dilution of antibody used in Western blots (e.g., 1:10,000).

7. Wash filters as in **step 3**.
8. Incubate with chemiluminescent detection reagents and expose to autoradiographic film. Exposure times are usually two to five times longer than for Western blots.
9. Align film to master plates. Pick positive plaques, replate, and perform secondary screenings, using **steps 2–8** (*see* **Notes 14–16**).

3.3. Peptide Overlay

Recent experiments with biotinylated anchoring inhibitor peptides have provided a nonradioactive assay for comparing RII-binding mutants. This technique has value for a number of systems in which the binding domains are small linear sequences (e.g., SH2 or SH3 recognition sequences, or small peptide hormone ligands). Peptides encompassing these binding regions can be purchased with a biotin conjugate to the α-amino group. Furthermore, the hydrophilic nature of the biotin moiety often increases the solubility of the peptide, as is the case for the HT31 peptide. This assay is a rapid nonradioactive technique that we have used to compare binding of different isoforms of RII, RII mutants, and truncations that affect AKAP binding.

1. Soak a nitrocellulose filter with 0.2 mL TBS, using a slot-blot manifold (e.g., Hybri-slot, BRL) (*see* **Note 17**).
2. RII proteins are immobilized by slot blotting the proteins in their native state onto nitrocellulose at a concentration range of 0.3–100 ng (*see* **Note 18**).
3. The membrane is blocked in TBS 5% dry milk, 1% BSA for 1 h.
4. A biotinylated RII-binding peptide (0.4 μM) is added to the blocking solution.
5. Incubate for 1–3 h at room temperature with gentle agitation (*see* **Note 19**).
6. The filter is washed three times with TTBS then probed with a 1:10,000 dilution of streptavidin-HRP in TTBS for 40 min.
7. Excess peptide is removed by washing in TTBS (three times).
8. The peptide–protein complex is detected using standard chemiluminescence methods.
9. The relative intensity of binding is measured by densitometric analysis of the autoradiogram. This is achieved by digitally scanning images into a computer and analyzing the band density using the NIH image program (*see* **Note 20**).
10. Numeric values correlating to band intensity can now be plotted against concentration to obtain binding curves (*see* **Note 21**).

3.4. Band-Shift Analysis

In contrast to many overlay techniques, band-shift analysis examines protein–protein interactions under nondenaturing conditions *(14,33)*. This method takes advantage of the observation that a protein complex migrates on a native polyacrylamide gel with a different mobility than its individual components (**Fig. 6**). Proteins are preincubated in binding buffer to allow complex forma-
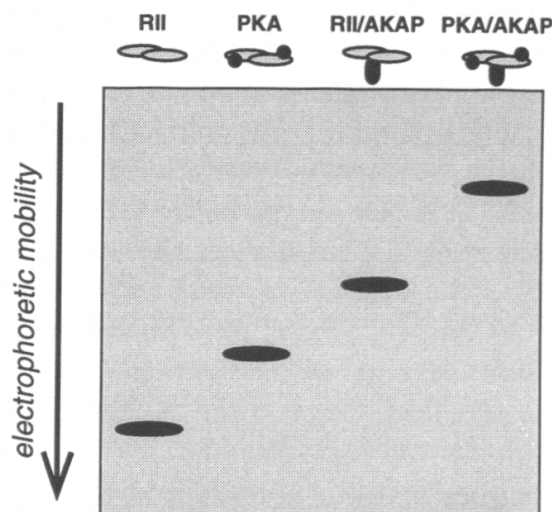
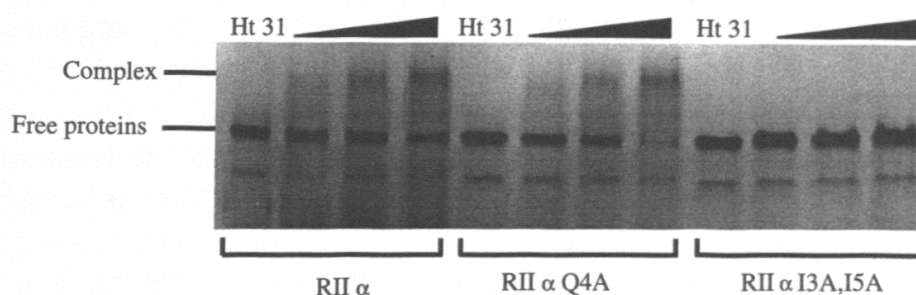Fig. 6. The band shift. A schematic diagram of the band-shift analysis.

Fig. 7. Mutation of isoleucines 3 and 5 impairs AKAP-binding as assessed by band-shift analysis. AKAP binding in solution was analyzed by the band-shift analysis using Ht 31 as a probe. Selected RIIα mutants (1 μ*M*) were incubated with aliquots of Ht 31 over a range of concentrations (0.33–1 μ*M*). The free and complexed proteins were separated on a 6% polyacrylamide gel: RIIα (panel 1), RIIα Q4A (panel 2), and RIIα I3A, I5A (panel 3). Detection was by protein staining with Fast stain™ (Zion Research).

tion, and, after dilution with sample buffer, free and complexed proteins are separated by electrophoresis. The migrating bands are detected by Coomassie blue staining or autoradiography.

Data in **Fig. 7** demonstrate the use of the band-shift analysis to compare the binding affinities of several RII mutants that contain point mutations in the AKAP-binding domain. For example, wild-type RII binds a recombinant AKAP fragment more readily than the double mutant RIIα I3A, I5A; a control mutation (Q4A) has no obvious effect upon binding. The sensitivity of this technique is limited by the high concentrations of protein required for detection of complexes, and equilibrium conditions are not maintained during electrophoresis.

1. Nondenaturing (ND) PAGE gels. ND-PAGE gels are prepared by the methods of **ref. 34**, except with the omission of SDS (*see* **Note 22**).
2. RII and AKAP (approx 3 µg) are incubated in binding buffer (5 m*M* HEPES, 10 m*M* DTT, 1 m*M* benzamidine, 100 m*M* KCl, and 0.01% Tween) at room temperature for 1 h.
3. Samples are diluted 1:1 in ND sample buffer (100 m*M* MOPS, 40% glycerol, 0.01% bromophenol blue), and are separated by electrophoresis at 30–50 mV at 16°C for ~3 h.
4. Stain the gel with a Coomassie-based stain for detection of protein bands (*see* **Note 23**).

3.5. Conclusions

Although the techniques that are described in this chapter are tailored for use in the analysis of RII–AKAP interactions, the approaches should be applicable to study of a wide range of protein–protein interactions. The speed and simplicity of these methods often make them a first choice for investigating the components of certain multiprotein complexes. However, one important point that must not be overlooked is that the overlay and band-shift methods we have described are at best semiquantitative. Therefore, it will often be necessary to more rigorously define many protein–protein interactions by more quantitative methods such as equilibrium dialysis, equilibrium centrifugation, or surface plasmon resonance measurements.

4. Notes

1. Unless otherwise stated, all procedures are performed at room temperature.
2. This step is important for correlation of nonspecific binding to abundant proteins within the extract.
3. Blots are often incubated overnight.
4. Dimerization between the labeled RII probe and immobilized RII monomer is weakly detected by the overlay, giving a band at 52–55 kDa. Although the ^{32}P overlay procedure seems to be specific for the detection of many RII-binding proteins, the C subunit of PKA is not detected by this procedure.
5. Unless otherwise stated, all procedures are performed at room temperature.
6. Incubation time is dependent upon the affinity and specificity of the antibody. Generally, we incubate with anti-RII antibodies (1: 20,000) for 3 h.
7. Multiple exposures are advisable to ensure that the signal is within the linear sensitivity range of the X-ray film.
8. Depending on the source of the antibody, endogenous RII will be detected in protein extracts screened by the nonradioactive overlay. Therefore, control filters should be screened in the absence of added RII protein to detect any background signals and false positives generated by the primary and secondary antibodies alone.
9. Immobilon cannot be used for this procedure, because the vacuum manifold will dry the filter, thereby destroying its protein-binding properties.

10. Air bubbles on the surface of the membrane prevent uniform adherence of the protein to the nitrocellulose.

11. AKAPs containing an endogenous, or engineered (**Subheading 3.1.4.**), PKA phosphorylation site can be labeled as described in **Subheading 2.1.1., steps 5–6**.

12. A maximal and minimal intensity control band is included on each image to normalize the analyzed images.

13. We have observed that purified AKAPs or RII directly immobilized on nitrocellulose, as compared to SDS-PAGE-separated samples, increases the sensitivity of detection 10- or 100-fold, respectively.

14. The bait protein used in these screening methods can be native, recombinant, or an isolated fragment thereof. We have also had success using biotinylated peptides as bait with streptavidin-HRP and chemiluminescent detection (*see* **Subheading 3.3.**).

15. For some interactions, nonmilk-based binding media, supplemented with additional factors (activators, divalent cations, and so on) may be required. When high background is problematic, we employ TBS with detergent (e.g., 0.5% Triton-X100) and high salt ($0.5 M$ NaCl) as the binding and washing buffer.

16. When using the nonradioactive screen (**Subheading 3.2.2.**), all positive plaques must also be screened in the absence of bait protein (**Subheading 3.2.2., step 2** omitted) to eliminate false positives retrieved by the antibodies alone (e.g., clones expressing cDNAs encoding the bait protein itself). This control step is easily performed on duplicate filters.

17. Immobilon cannot be used for this procedure, because the vacuum manifold will dry the filter, thereby destroying its the protein-binding properties.

18. Make sure to prevent bubbles from forming on the surface of the membrane, because the protein solution is applied to the nitrocellulose.

19. The probing time can be as short as 1 h, but optimal binding needs to be empirically determined for each peptide.

20. A maximal and minimal intensity control band is included on each image to normalize the analyzed images.

21. The peptide overlay was found to be linear for interaction with AKAPs in a very narrow range (80–15 ng). RII proteins can also be separated by SDS-PAGE, transferred to a hydrophobic membrane, and probed with the biotinylated peptides. We have observed that the latter method has a decreased sensitivity of up to 100-fold.

22. The optimal gel concentration needs should be determined empirically for the proteins being used. For example, separation of RII/AKAP complexes is optimally achieved with 6% (w/v) polyacrylamide separating gels and a 4% (w/v) stacking gel.

23. Several factors contribute to good quality of protein bands. These are:
    a. Prepare fresh acrylamide stock.
    b. Freshly prepare binding buffer.
    c. Separating gel should be rinsed free of isobutanol, if used, thoroughly before pouring the stacking gel.
    d. Gel should be kept at 16°C during the separation.

Acknowledgments

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