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Localization of the cAMP-dependent Protein Kinase to the Postsynaptic Densities by A-Kinase Anchoring Proteins

CHARACTERIZATION OF AKAP 79*

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Postsynaptic densities (PSD) are a network of proteins located on the internal surface of excitatory synapses just inside the postsynaptic membrane. Enzymes associated with the PSD are optimally positioned to respond to signals transduced across the postsynaptic membrane resulting from excitatory synaptic transmission or neurotransmitter release. We present evidence suggesting that type II cAMP-dependent protein kinase (PKA) is anchored to the PSD through interaction of its regulatory subunit (RII) with an A-Kinase Anchor Protein (AKAPs). A cDNA for the human RII-anchoring protein, AKAP 79, was isolated by screening an expression library with radiolabeled RII. This cDNA (2621 base pairs) encodes a protein of 427 amino acids with 76% identity to bovine brain AKAP 75 and 93% identity to a carboxyl-terminal RII-binding fragment of murine brain AKAP 150. A bacterially expressed 92-amino acid fragment, AKAP 79 (335–427) was able to bind RIIα. Disruption of secondary structure by site-directed mutagenesis at selected residues within a putative acidic amphipathic helix located between residues 392 and 408 prevented RII binding. Immunological studies demonstrate that AKAP 79 is predominantly expressed in the cerebral cortex and is a component of fractions enriched for postsynaptic densities. AKAP antisera strongly cross-react with a 150-kDa protein in murine PSD believed to be AKAP 150. Co-localization of the type II PKA in purified PSD fractions was confirmed immunologically by detection of RII and enzymologically by measuring cAMP-stimulated phosphorylation of the heptapeptide substrate Kemptide. Approximately 30% of the PSD kinase activity was specifically inhibited by PKI 5–24 peptide, a highly specific inhibitor of PKA. We propose that AKAP 79 and AKAP 150 function to anchor the type II PKA to the PSD, presumably for a role in the regulation of postsynaptic events.

In recent years it has become apparent that a significant proportion of the type II cAMP-dependent protein kinase (PKA)[1] is compartmentalized in specific sites in the cell. Co-localization of PKA with its substrates is a potential mechanism to explain how a multifunctional kinase with a broad substrate specificity, such as PKA, can mediate the precise biochemical events associated with its activation by individual hormones (1). Upon activation by cAMP, the catalytic (C) subunit is released from the regulatory subunit (R) dimer (2, 3), and preferentially phosphorylates substrates within that vicinity. To maintain the type II PKA in specific cellular compartments, the R subunit (RII) interacts with specific RII-anchoring proteins (4, 5).

Several RII-anchoring proteins have been identified and characterized. Cytoskeletal attachment of type II PKA occurs through interactions between RII and microtubule-associated protein 2 (MAP 2) (6). The site on MAP 2 that contacts RII has been identified as a 31-residue domain in the amino-terminal region of the molecule (7, 8). RII also associates with a bovine brain calmodulin-binding protein designated P-75 (4, 9). Several P-75 analogs, ranging in size from M_r 60,000 to 150,000 have been reported in different species and may represent members of a family of structurally related RII-anchoring proteins (10). Partial cDNA clones encoding the RII-binding domains for two members of this family, P-150 and P-75, have been isolated from bovine and murine brain libraries, respectively (10, 11). A 96-amino acid fragment of a human RII-binding protein that migrates at 79 kDa has recently been published (12). This fragment is 92% identical with the corresponding regions of P-75 and P-150. Rubin and colleagues (12) have renamed this family of RII-binding proteins as A-Kinase Anchor Proteins (AKAP 75, AKAP 79, and AKAP 150). Deletion mutants lacking the last 26 residues of AKAP 75 or the last 15 residues of AKAP 150 do not bind RII, suggesting that sequences at the extreme carboxyl terminus of each molecule are required for anchoring (10, 11). However, comparison of these sequences with the RII-binding region of MAP 2 reveals no striking homology, suggesting that each protein may contain a conserved secondary structure responsible for binding. This hypothesis is strengthened by the identification of a 14-residue sequence in a fourth RII-anchoring protein, called Ht 31, which was similar to sequences in the RII-binding domains of MAP 2, AKAP 75, and AKAP 150 (13). Computer-generated predication of secondary structure performed on all four proteins identified potential amphipathic helices in each molecule. Two lines of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M90359.

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; MAP 2, microtubule-associated protein 2; AKAP, A-kinase anchor protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; TBS, Tris-buffered saline; PCR, polymerase chain reaction; PSD, postsynaptic density; SDS, sodium dodecyl sulfate.

evidence suggest that an acidic amphipathic helix forms the RII-binding in Ht 31. Site-directed mutagenesis designed to disrupt secondary structure within the putative binding helix destroys RII interaction (13), and a 24-residue peptide spanning the amphipathic helix region binds RIIα or the type II PKA holoenzyme with nanomolar affinity (14).

In this report we describe the cloning and characterization of a full-length cDNA for AKAP 79, a human RII-anchoring protein that belongs to the AKAP 75/150 family of anchoring proteins. Immunological studies with AKAP 79 antisera demonstrate that all three family members are predominantly expressed in the cerebral cortex and specifically localized to the postsynaptic densities of the forebrain. Functional analyses of the recombinant protein expressed in *Escherichia coli* have demonstrated that AKAP 79 is a substrate for PKA and a calmodulin-binding protein. Furthermore, the RII-binding domain of AKAP 79 includes a putative amphipathic helix region whose conformation must be unaltered for binding.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of AKAP 79—A human thyroid cDNA library in λ gt 11 was screened with radiolabeled RII to detect plaques expressing RII-binding proteins. Filters were soaked in 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and placed on bacterial lawns for 4 h at 37 °C to induce expression of the β-galactosidase fusion proteins. Duplicate filters were prepared by the same procedure. All filters were blocked by incubation in 50 mM Tris, pH 7.5, 150 mM NaCl (TBS), and 1% powdered milk solution for 4 h at 4 °C. Filters were probed with [^{32}P]RII overnight in TBS, 1% powdered milk solution at room temperature. To avoid nonspecific binding, a 10-fold molar excess of RII (Δ1–23), a mutant that does not bind anchoring proteins, was included in the probe solutions. Filters were washed three times in TBS, 1% Tween 20, and positive colonies were visualized by autoradiography. DNA from positive colonies was purified; cDNA inserts were cut out of λ phage by digestion with *Eco*RI and subcloned into Bluescript KS (Stratagene) vectors for sequencing. Nucleotide sequencing was performed on one clone, designated Ht 21, by the Sanger dideoxynucleotide method (15) using a strategy that required the synthesis of unique oligonucleotide primers.

Expression of AKAP 79 and Fragments—A 1299-base pair insert encompassing the entire coding region of AKAP 79 was amplified by polymerase chain reaction (PCR) in a Coy tempcycler, using Bluescript Ht 21 as a template. PCR primers were designed to create an *Nco*I site at the 5′ end and a *Bam*HI site at the 3′ end of the insert. After digestion with both restriction enymes, the 1291-base pair *Nco*I-*Bam*HI fragment was inserted into the bacterial expression vector pET 11d (Novagen). The carboxyl-terminal 92 amino acids of AKAP 79 were expressed in the *E. coli* expression vector pETkfc (17), which is an adaptation of the T7 polymerase system reported by Studier *et al.* (17). A 298-base pair fragment encoding the 3′ end of the AKAP 79 cDNA was amplified by PCR as described above. As before, PCR primers were designed to create an *Nco*I site at the 5′ end and a *Bam*HI site at the 3′ end of the insert. After digestion with both restriction enzymes, the 286-base pair *Nco*I-*Bam*HI fragment was inserted into the bacterial expression vector, pETkfc. Expression of AKAP 79 (335–427) kfc was performed as described by Studier *et al.* (17), and the expressed protein was purified by affinity chromatography on calmodulin-agarose (16). PCR products were sequenced to confirm that they contained the authentic sequence.

Site-directed Mutagenesis of AKAP 79 (335–427)—Mutants were designed to disrupt the putative secondary structure of AKAP 79 (335–427) by substituting proline residues (CC*X*) for selected amino acids. Single point mutations in AKAP 79 (335–427) were introduced at Leu-392 (TTA), Ala-396 (GCC), Val-400 (GTC), Gln-405 (CAG), Ile-408 (ATA), Val-412 (GTT), Met-415 (ATG), and Asp-418 (GAT) using mutagenic oligonucleotides of 21 bases. All mutagenesis was performed by the PCR method of Nelson and Long (18), using a Coy tempcycler. This method employed four oligonucleotide primers. One oligonucleotide contained the base mismatches to direct the mutagenesis; the other three oligonucleotides allowed the selective amplification of the mutated sequence. The primers for PCR mutagenesis are: A, the mutagenic primer; B, a reverse primer composed of a 20-nucleotide 3′ sequence complementary to a site in pET 11d downstream of the coding sequence and a unique 20-nucleotide 5′ sequence (GGGGTACTAGTAACCCGGGCCGGGCTTTGTTAGCAGCCGG); C, a 20-nucleotide forward primer to the T7 promoter and; D, a reverse primer complimentary to the 20-nucleotide unique 5′ sequence of primer B (CCGGCTGCTAACAAAGCCCG). Thirty cycles of PCR were performed with 100 pmol of primers A and B using 1 fmol of *Hin*dIII linearized pETkfc AKAP 79 (335–427) as the template. The expected product was extracted from a 1.5% agarose gel and used as a primer for the second step. The second step reaction included the first reaction product and the *Hin*dIII linearized pETkfc AKAP 79 (335–427). This PCR reaction was run for two cycles of 3 min at 95 °C, 2 min at 37 °C, and 2 min at 72 °C. Primers C and D (100 pmol each) were then added, and an additional 30 cycles of PCR were performed. The final 488-base pair product was phenol-extracted, ethanol-precipitated, and cut with *Nco*I and *Bam*HI. The 297-base pair mutant *Nco*I-*Bam*HI fragments were inserted into pETkfc and transfected into competent *E. coli* BL 21 (DE 3) cells for expression of the mutant protein. PCR products were sequenced to confirm the mutant sequence.

Preparation of Synaptosomes and Postsynaptic Densities (PSDs)—Synaptosomes were purified from rat forebrains according to the method of Rich *et al.* (19) and kindly provided by Drs. S. E. Tan and T. R. Soderling (Vollum Institute). PSDs were prepared from a crude synaptosomal fraction that was exposed to 1% Triton X-100. Following detergent solubilization to remove plasma membranes and synaptic vesicles, the PSDs were purified by a series of discontinuous sucrose gradients. Human cerebral cortex was obtained from the Oregon Health Sciences University Research Brain Repository and Division of Neuropathology, and the PSD was purified by the method of Carlin *et al.* (20).

Protein Kinase Assays—The PKA activity of PSD fractions was determined by the filter paper assay of Corbin and Reimann (21) with modifications described by Scott *et al.* (22). PKA activity was specifically inhibited by a 20-residue peptide inhibitor (TTYADFIASGRTGRRNAIHD) derived from residues 5 to 24 of the heat-stable inhibitor of the cAMP-dependent protein kinase (23, 24).

RII Overlay Procedure—The RII overlay procedure was performed by the method of Lohmann *et al.* (5) with modifications documented by Scott *et al.* (22).

Protein Bandshift Analysis—Bandshift analysis was performed by a modification to the method described by Carr *et al.* (13). Partially purified AKAP 79 was incubated with ^{125}I-calmodulin prior to electrophoresis on a 6% (w/v) nondenaturing polyacrylamide gel with 2 mM Ca^{2+} included in all buffers and gel mixtures. After electrophoresis, the gel was dried and the migration of ^{125}I-calmodulin complexes was detected by autoradiography.

Other Methods—Peptides were synthesized by the Oregon Health Sciences University peptide synthesis facility, and HF cleavage from the resin was as described by Scott *et al.* (24). Recombinant RIIα was phosphorylated as described by Scott *et al.* (22). Western blots were performed by the methods of Towbin *et al.* (25). Human tissue was collected 8 h postmortem and kindly supplied by Dr. Geoffrey Murdoch (Oregon Health Sciences University). Detection of immunoreactive proteins on immunoblots was achieved by using the ECL Western blotting detection system (Amersham Corp.). Calmodulin was radiolabeled with ^{125}I by the Chloramine-T method (26). Antibodies against recombinant AKAP 79 were produced by Bethyl Laboratories, Inc. (Montgomery, TX).

RESULTS

Cloning of a Human Thyroid PKA-anchoring Protein, AKAP 79—Using a modified RII overlay procedure, a human thyroid λ gt 11 expression library was screened for RII-binding proteins. Six positive clones were identified from 5×10^5 plaques. Each positive clone was subcloned into the *Eco*RI site of Bluescript KS vectors for sequence analysis (as described under "Experimental Procedures"). Detailed restriction mapping and partial sequence analysis determined that three cDNAs were identical. One of these clones, designated Ht 21, was sequenced on both strands. The nucleotide sequence (2621 base pairs) and predicted primary structure of the Ht 21 protein is presented in Fig. 1. The Ht 21 cDNA contained an unusually long 5′-untranslated region followed by an open reading frame of 1281 base pairs that encoded a protein of 427 amino acids. The ATG at position 1299 was

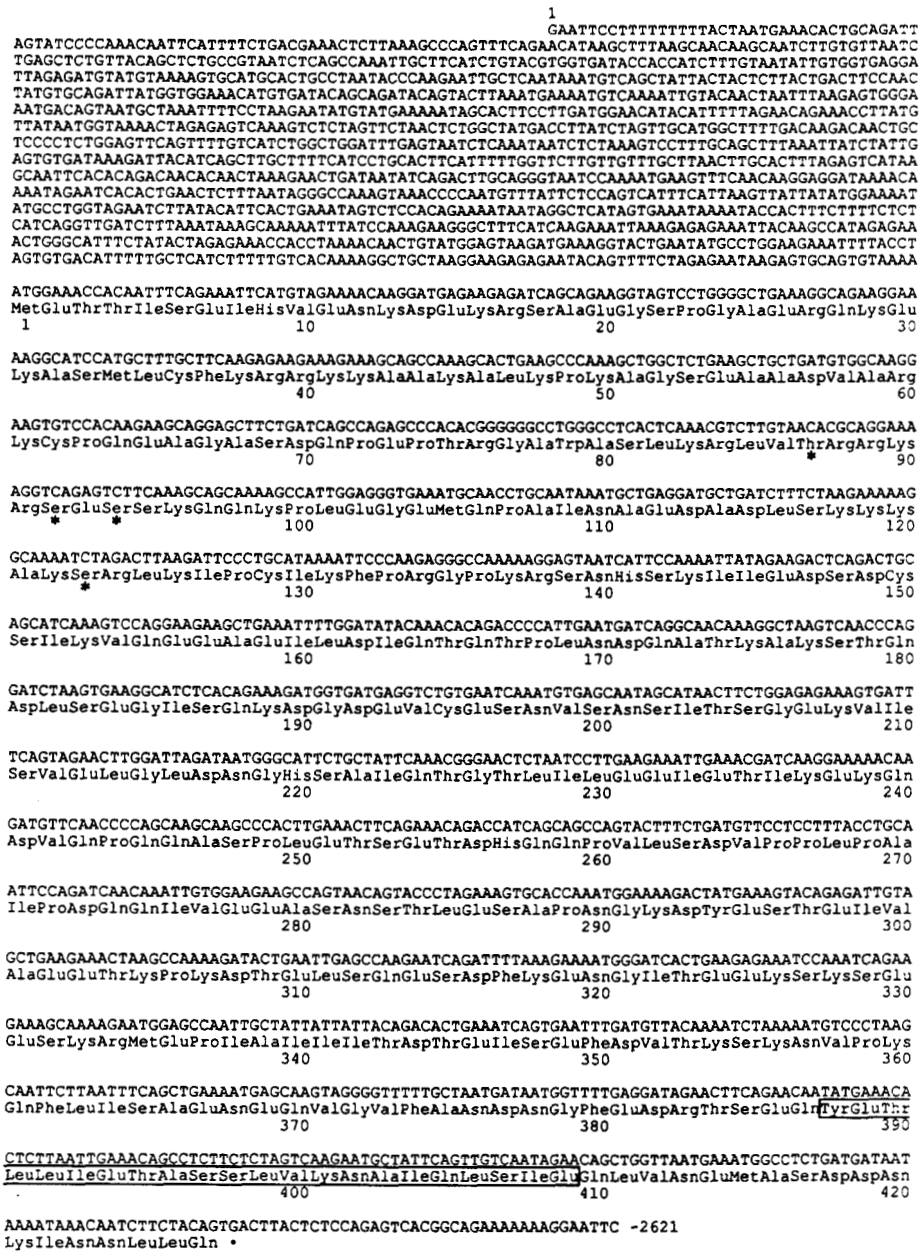

FIG. 1. **The sequence of AKAP 79 cDNA.** The nucleotide sequence (*upper line*) and deduced amino acid sequence (*lower line*) of a cDNA encoding the human AKAP 79 are shown. The *boxed region* indicates the RII-binding domain. Consensus sites for the cAMP-dependent protein kinase (*asterisks*) are indicated below the target amino acids.

believed to be the initiation codon since it is the first ATG in frame with the coding region, and the surrounding nucleotide sequence contains several determinants that are in optimal context for the initiation of translation (27). This was confirmed when the Ht 21 coding region was cloned into pET 11d and produced a protein of identical size to human cerebral cortex AKAP 79.

The Ht 21 sequence is 76% identical to bovine AKAP 75 and 93% identical to residues 358–464 of murine AKAP 150 (Fig. 2) and differs by only one amino acid to the partial sequence of AKAP 79 previously published (12). To conform with the nomenclature of Rubin and colleagues (12), Ht 21 has been renamed AKAP 79. The numbers refer to the migration of the native proteins on SDS-polyacrylamide gels (Fig. 3*B*). AKAP 79 has no overall homology with any other sequences in the GenBank™ or PIR sequence data bases.

Expression of AKAP 79 and Characterization of the RII-binding Domain—AKAP 79 and the carboxyl-terminal fragment, AKAP 79 (335–427), were expressed in *E. coli* using the Studier vector system (17). A prominent protein band of M_r 10,000 was detected in cell lysates of IPTG-induced *E. coli* cells containing pETkfc AKAP 79 (335–427). AKAP 79 (335–427) expression was maximal at 2½ h after IPTG induction and constituted approximately 25% of the total soluble protein, as assessed by SDS-polyacrylamide gel electrophoresis (Fig. 3*A*). In contrast, full-length AKAP 79 was produced at much lower levels (Fig. 3*A*) and was predominantly located in the particulate fraction. Since AKAP 79 (335–427) and the full-length molecule both bind RIIα as assessed by RII overlay (Fig. 3*B*), it was concluded that the carboxyl-terminal 92 amino acids of AKAP 79 include the RII-binding domain.

We have previously proposed that regions of conserved secondary structure that contain an acidic amphipathic helix motif form the RII-binding domain in anchoring proteins (13). Computer-aided analysis of the AKAP 79 sequence identified a putative amphipathic helix located between residues 392 and 405, which has sequence similarity to another RII-anchoring protein, Ht 31. Therefore, a family of point mutants was produced in AKAP 79 (335–427) to determine if a helical conformation is required for RII binding. Within an

```
                                25                                50
AKAP79      METTISEIHVENKDEKRSAEGSPGAERQKEKASMLCFKRRKKAAKALKPK
AKAP75      ..I.V...Q..S...T....VR.QD...E.................M...
                                75                               100
AKAP79      AGSEAADVARKCPQEAGASDQPEPTRGAWASLKRLVTRRKRSESSKQQKP
AKAP75      .S.K...A.K...P..R.....QRPG...D.I.................
                               125                               150
AKAP79      LEGEMQPAINAEDADLSKKKAKSRLKIPCIKFPRGPKRSNHSKIIEDSDC
AKAP75      FKAKL.SE......NP.................SK.E............R
                               175                               200
AKAP79      SIKVQEEAEILDIQTQTPLNDQATKAKSTQDLSEGISQKDGDEVCESNVS
AKAP75      .V...-...N.VTK...QSD.....S..P..VR.DV...GD........N
                               225                               250
AKAP79      NSITS-GEKVISVELGLDNGHSAIQTGTLILE-EIETIKEKQDVQPQQAS
AKAP75      .....P.........E..M......R......KDT.MLE...SI...HV.
                               275                               300
AKAP79      PLETSETDHQQPVLSDVPPLPAIPDQQIVEEASNSTLESAPNGKDYESTE
AKAP75      ...A.D.EQEL..G.E...SS.V.....L...R.GV...G.DW.EH..R.
                               325                               350
AKAP79      IVAEETKPKDTELSQESDFKENGITEEKSKSEESKRMEPIAIIITDTEIS
AKAP75      ..V..S..........L..Q..E..A..P.P....................
AKAP150 (358-464)                .VDA..PR....................
                               375                               400
AKAP79      EFDVTKSKNVPKQFLISAENEQVGVFANDNGFEDRTSEQYETLLIETASS
AKAP75      ....K.......P....I...........S...G.................
AKAP150     ....K............M...........SD..G.................
                               425
AKAP79      LVKNAIQLSIEQLVNEMASDDNKINNLLQ
AKAP75      .......................T...R..
AKAP150     ......E..V.......V.E..Q..T.F.
```

FIG. 2. **Sequence comparison of AKAP 79 with AKAP 75 and AKAP 150.** The AKAP 79 sequence (*top*) is aligned with the predicted coding sequence of the bovine AKAP 75 gene (*middle*) and the COOH-terminal region (residues 358–464) of murine AKAP 150 (*bottom*). Sequences are aligned with gaps (–) to show maximum identity. Amino acids identical with AKAP 79 are indicated by *dots* below the AKAP 79 sequence.

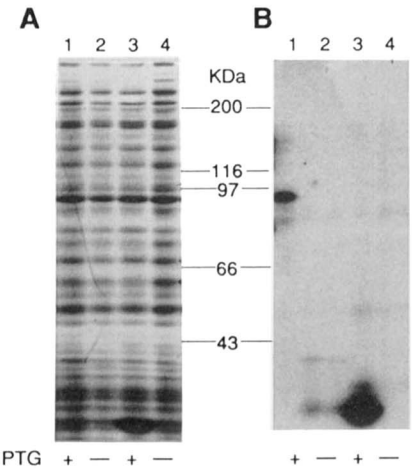

FIG. 3. **Binding of RII to bacterially expressed AKAP 79 and AKAP 79 (335–427).** Bacterial extracts (50 μg of protein) were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to Immobilon for analysis by RII overlay binding assay. Protein bands were detected by staining with Coomassie Brilliant Blue (*panel A*), and [^{32}P]RIIα binding was detected by autoradiography (*panel B*). Bacterial extracts from cells induced with 1 mM IPTG (*lanes 1* and *3*) are compared with non-induced cells (*lanes 2* and *4*). *E. coli* cells containing AKAP 79 in plasmid pET 11d are shown in *lanes 1* and *2; E. coli* cells containing AKAP 79 (335–427) in plasmid pET 11d kfc are shown in *lanes 3* and *4;* molecular mass markers are indicated. Extracts were analyzed for [^{32}P]RIIα binding by a solid-phase assay using a modified overlay procedure (21).

extended region of sequence that encompassed the predicted amphipathic helix of AKAP 79, selected amino acids were mutated to proline (Fig. 4*A*). The introduction of proline can result in a 20° bend in an α-helix conformation and often disrupts the secondary structure of proteins. Mutants were expressed in the pETkfc vector system, and each protein was analyzed for the ability to bind RII by solid-phase overlay assay (Fig. 4, *B* and *C*).

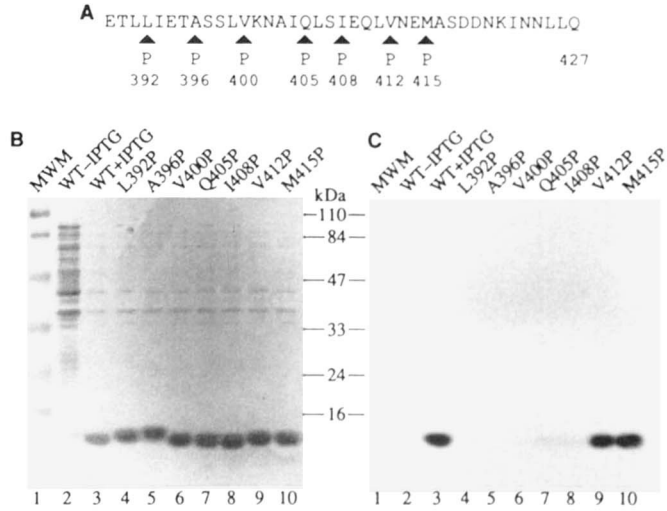

FIG. 4. **Disruption of RII binding activity in AKAP 79.** *A*, the carboxyl-terminal sequence of AKAP 79 with the amino acid positions mutated to prolines as indicated. Mutant AKAP 79 (335–427) proteins were partially purified from bacterial extracts and separated by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and electrophoretically transferred to an Immobilon membrane. The blot was stained with Coomassie Brilliant Blue (*B*) and analyzed for RII binding by autoradiography (*C*) after incubation with ^{32}P-labeled RIIα. *Lane 1* contains the molecular weight markers (*MWM*) with sizes indicated between the gels. Extracts of AKAP 79 (335–427) wild type (*WT*), grown in the presence (*lane 2*) and absence (*lane 3*) of 1 mM IPTG, are indicated. The various proline mutations are in the *lanes* indicated.

The introduction of proline at residues 392, 396, 400, 405, or 408 prevents or severely diminishes RII binding (Fig. 4*C*). These residues are located within, or immediately adjacent to, the predicted α-helix. Therefore, mutational analysis suggests that disruption of the secondary structure between residues 392 and 408 of AKAP 79 prevents interaction with RIIα. Control mutations outside the putative amphipathic helix at residues 412 and 415 had no qualitative effect on RII interaction (Fig. 4*C*), demonstrating that substitution with proline does not destabilize the overall conformation of the protein. In order to confirm that this region is a determinate for RII binding, a 24-residue peptide, Tyr-Glu-Thr-Leu-Leu-Ile-Glu-Thr-Ala-Ser-Ser-Leu-Val-Lys-Asn-Ala-Ile-Gln-Leu-Ser-Ile-Glu-Gln-Leu-, which encompasses the putative amphipathic helix, was synthesized. AKAP 79 (388–409) peptide blocks RII interaction with AKAP 79 and other anchoring proteins as assessed by overlay assay (Fig. 5). These results support our hypothesis that an acidic amphipathic helix is a conserved binding motif for RII/AKAP interaction.

Calmodulin Binding and PKA Phosphorylation of AKAP 79—Previous studies have shown that AKAP 75 is a calmodulin-binding protein (4). To determine if calmodulin binds to AKAP 79, the two proteins were incubated together and then analyzed by bandshift gel electrophoresis. A complex of AKAP 79 and ^{125}I-calmodulin was detected by a shift in migration of labeled calmodulin on autoradiography (Fig. 6, *lane 2*). Since AKAP 79 (335–427) is unable to bind calmodulin, it was concluded that the amino-terminal regions of AKAP 79 are responsible for interaction with calmodulin. Likewise, full-length AKAP 79 binds to calmodulin-agarose in a Ca^{2+}-dependent manner but AKAP 79 (335–427) does not (data not shown).

Co-localization of AKAP 79, AKAP 150, and PKA to the Postsynaptic Densities—Recombinant AKAP 79 was purified by affinity chromatography on calmodulin-agarose and used to raise antisera in rabbits. Immunoblots of several tissues

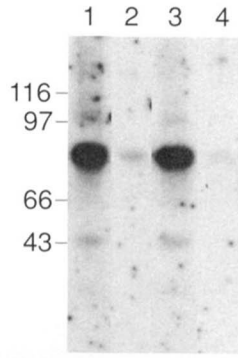

FIG. 5. **Competition of RIIα/AKAP 79 interaction with AKAP (388–409) peptide.** Protein extracts of human cerebral cortex (25 μg) were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. After electrophoresis, the proteins are electrophoretically transferred to Immobilon. Samples were analyzed for [^{32}P]RIIα binding by a solid-phase assay using a modified overlay procedure (21). Samples were incubated with [^{32}P]RIIα alone (*lanes 1* and *3*) or with [^{32}P]RIIα that had been preincubated for 15 min with either 25 or 60 μM AKAP 79 (388–409) peptide (*lanes 2* and *4*, respectively). Molecular weight markers are indicated. The stock peptide solution contained dimethyl sulfoxide to aid in solubilizing the peptide; as a control, equal volumes of dimethyl sulfoxide without the peptide were added to *lanes 1* and *3* (0.6 and 1.5% dimethyl sulfoxide, respectively).

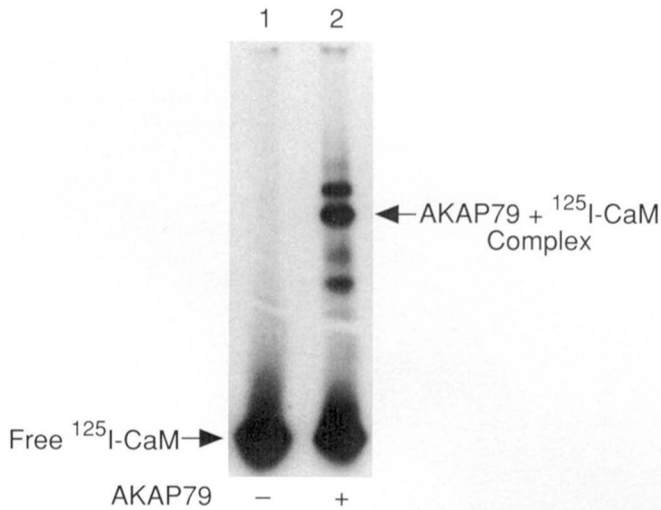

FIG. 6. **Binding of AKAP 79 to calmodulin.** The ability of AKAP 79 to interact with calmodulin was assessed by nondenaturing electrophoresis. AKAP 79 (5 μg) and ^{125}I-radiolabeled calmodulin (*^{125}I-CaM*) (0.1 μg) were incubated for 1 h at 20 °C and separated by nondenaturing electrophoresis on a 6% (w/v) polyacrylamide gel with 2 mM Ca^{2+} added to all buffers. The AKAP 79-calmodulin complex was detected by autoradiography. *Lane 1* contains ^{125}I-radiolabeled calmodulin alone and *lane 2* the ^{125}I-radiolabeled calmodulin-AKAP 79 complex.

were analyzed with polyclonal anti-AKAP 79 antisera at a dilution of 1:2000. A 79-kDa protein was recognized in human cerebral cortex and, to a lesser extent, in human brain white matter and thyroid (Fig. 7*A*). These bands were absent when parallel blots were analyzed with pre-immune serum from the same rabbit. The human thyroid protein (Fig. 7*A*, *lane 5*) migrates at a slightly slower mobility, which may be due to post-translocation modification; however, recombinant AKAP 79 migrates at a rate equal to the human cerebral cortex protein (Fig. 7*B*, *lanes 1* and *2*). Anti-AKAP 79 antisera also detect cross-reacting proteins of 75 and 150 kDa in bovine cerebral cortex and murine brain extracts (Fig. 7*A*). These data further support the notion that AKAP 79 is a human homolog of the AKAP75/150 family of RII-anchoring proteins. Other bands of lower molecular weight were also detected with the anti-AKAP 79 antibody in the human and bovine brain extracts; however, these proteins do not bind to RII in solid-phase overlay assays (Fig. 7*B*), and their significance is unclear. A survey of other tissues including human cerebellum, adrenal, liver, skeletal muscle, heart, lung, liver, and kidney and bovine skeletal muscle, heart, thyroid, lung, liver, and pituitary was unable to detect any other protein that cross-reacted with anti-AKAP 79.

Immunoreactive proteins of 79 and 150 kDa were detected with the anti-AKAP 79 antisera in the subcellular fractions of synaptosomes and purified PSDs from human and murine tissue, respectively (Fig. 7*A*). The 150-kDa band was enriched severalfold in the murine PSD compared with the synaptosome (Fig. 7*A*). The human PSD was also enriched for AKAP 79, but to a lesser extent than the murine counterpart. Parallel blots were analyzed for RII-binding proteins by the solid-phase overlay procedure. Prominent bands of 79 and 150 kDa were detected in human and murine fractions, respectively (Fig. 7*B*, *lanes 7–10*). In both cases, the 79- and 150-kDa bands were the major RII-anchoring proteins detected in purified human and murine PSD fractions (Fig. 7*B*).

We reasoned that if an RII-anchoring protein is a component of the PSD, the type II PKA should be co-localized there also. Therefore, immunoblots were probed with polyclonal anti-murine RII antisera and detected RII in both synaptosome and purified PSD fractions (Fig. 7*C*). The type I R subunit was not detected in either the synaptosomes or the PSD when filters were probed with anti-murine RI antisera (data not shown). Furthermore, incubation of PSD extracts in the presence of 10 μM cAMP increased the phosphorylation of the synthetic PKA substrate peptide Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) compared with control (Fig. 8). Approximately 30% of Kemptide-phosphorylating activity was inhibited by PKI (5–24) peptide, a specific inhibitor of PKA (23), indicating that the C subunit of the PKA is also present in the PSD (Fig. 8). Through the combination of immunological and enzymological assays, we concluded that AKAP 150 and the type II PKA are co-localized in the murine PSD.

DISCUSSION

In this report we describe the cloning and functional characterization of the human AKAP 79. Structural similarity and immunological cross-reactivity with two other RII-anchoring proteins, AKAP 75 and AKAP 150 (formerly called P-75 and P-150), suggest that AKAP 79 is their human homolog. AKAP 79 is 76% identical to AKAP 75, and residues 325–427 of AKAP 79 are 93% identical to a corresponding region of AKAP 150 (Fig. 2). The AKAP 75/150 family of RII-anchoring proteins is predominantly expressed in the central nervous system (4, 9–12). A survey of nine human and eight bovine tissues detected AKAP 79 and its homologs in only the brain and human thyroid. However, the cDNA for AKAP 79 was isolated from a human thyroid library, suggesting that either the protein also has a specialized function in the thyroid or the tissue used for cDNA synthesis contained low levels of neuronal tissue.

The calculated molecular weight of AKAP 79 is 47,085, although the protein migrates with a mobility of M_r 79,000 on SDS-polyacrylamide gels (Fig. 3). This discrepancy in apparent molecular weight must be a characteristic of the amino-terminal portion of the protein since the carboxyl-terminal RII-binding fragment AKAP 79 (335–427) migrates with the expected molecular weight of 10,000 on SDS-polyacrylamide gels (Fig. 3). Hirsch *et al.* (12) have suggested that

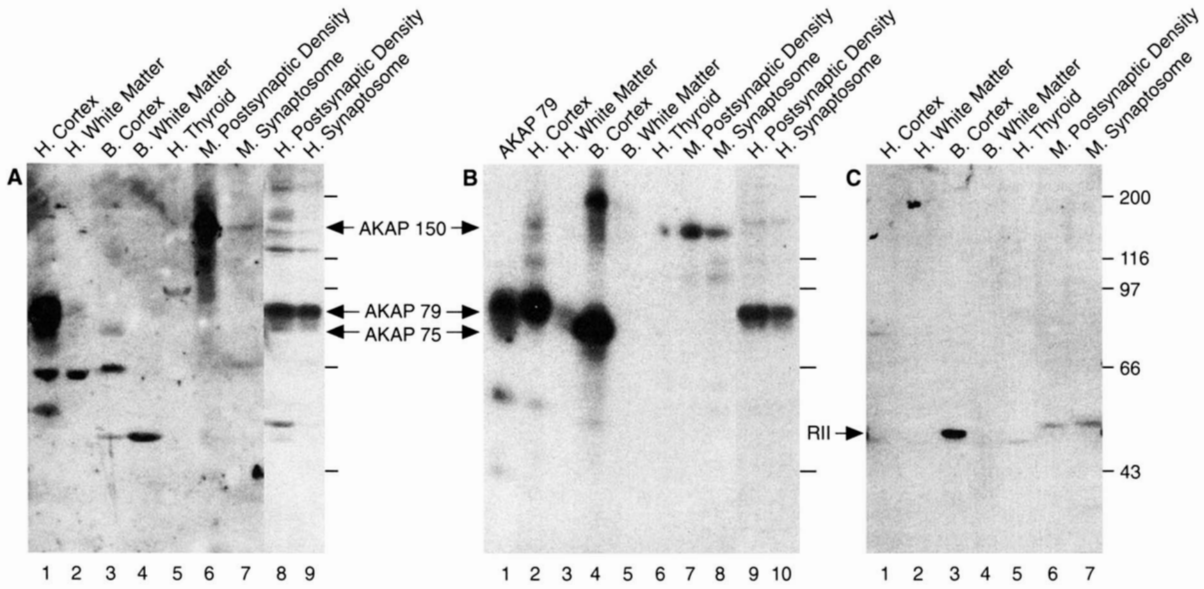

FIG. 7. **Immunological detection of AKAP 75, 79, and 150 and co-localization of AKAP 79 and 150 with RII in the postsynaptic densities.** Protein extracts of various issues (5–25 μg) were separated by gel electrophoresis on a 10% (w/v) SDS-polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to Immobilon for Western analysis with anti-AKAP 79 antisera (*A*), anti-murine RII antisera (*C*), or analysis of RII binding by RII overlay with ^{32}P-labeled RIIα (*B*). The tissue source of each sample is marked above the *lane* (*H.*, human; *B.*, bovine; *M.*, murine); migration positions of AKAP 75, AKAP 79, AKAP 150, and RII are indicated. Molecular weight markers are indicated on each *panel*.

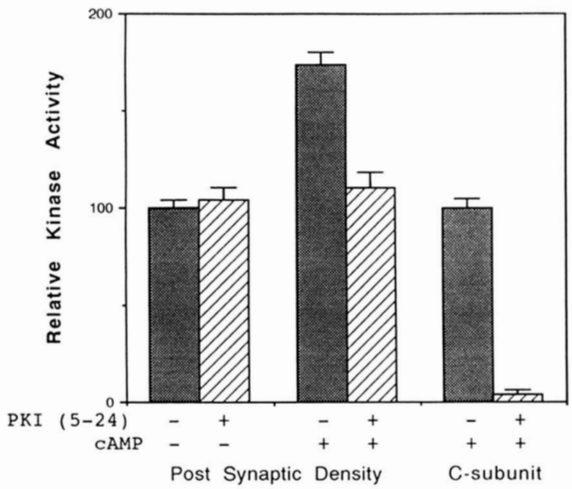

FIG. 8. **Measurement of cAMP-dependent protein kinase activity in the postsynaptic densities.** Purified fractions of the PSD (0.1 μg) or purified C subunit of the PKA were analyzed for protein kinase activity using the synthetic heptapeptide Kemptide (Leu-Arg-Arg-Ala-**Ser**-Leu-Gly) as a substrate. Samples were analyzed by the filter paper procedure of Corbin and Reimann (21), and incorporation of radioactivity was measured by Cerenkov counting. Kinase activity is expressed in relative units. The addition of cAMP (100 μM) or the PKI (5–24) peptide (20 μM), a specific inhibitor of the catalytic subunit of PKA, is indicated.

the discrepancy between the calculated and apparent M_r of AKAP 75 (75,000) is due to the highly acidic nature of a central region of the molecule (residues 180–320), which binds poorly to SDS, thereby causing the protein to migrate with reduced mobility. Given the considerable sequence similarity between both proteins, this explanation is most likely true for AKAP 79 also.

We have previously demonstrated that disruption of a putative amphipathic helix region within the RII-anchoring protein, Ht 31, prevents binding to RIIα (13). Synthetic peptides that encompass the amphipathic helix region of Ht 31 adopt a helical conformation, as assessed by circular dichroism, and bind RII or the type II PKA with nanomolar affinity (14). Based on these findings and the identification of a similar region in MAP 2, we have proposed that acidic amphipathic helices may be a common motif that can be used to identify RII-binding sites in other RII-anchoring proteins. Consistent with this hypothesis, a putative amphipathic helix region was identified at the extreme carboxyl terminus of AKAP 79, between residues 392 and 405 (Fig. 4*A*). Moreover, this sequence is highly conserved in AKAP 75 and AKAP 150 (Fig. 2) and shares a 43% identity with the corresponding region of Ht 31 (residues 494–507). Disruption of the secondary structure by substitution of proline at selected sites within the putative helix of AKAP 79 prevents RII binding, whereas similar substitutions outside the region have no effect (Fig. 4*C*). These results are consistent with previous studies showing that secondary structure is an important determinant for RII-anchoring protein interaction. Additionally, RII interaction with AKAP 79 was blocked by a 24-residue peptide that encompasses the putative amphipathic helix region. To aid solubility, this peptide was dissolved in buffers containing 0.6% dimethyl sulfoxide (final concentration) when used in RII overlay procedures (Fig. 5). Unfortunately, extreme insolubility of AKAP 79 (388–409) peptide in any buffers compatible with spectral analysis prevented the use of circular dichroism to determine the helical content of the peptide.

The amino-terminal region of AKAP 79 is rich in basic amino acids (Fig. 2). In fact, 47 of the 59 basic residues in the protein are located in the first 150 residues (Fig. 2). The overall positive charge in this region may function to attach AKAP 79 to subcellular structures via electrostatic interactions. This same region may contain a calmodulin-binding site. Although there is little or no conservation in primary structure, calmodulin binding sequences are identified as regions with an abundance of basic and hydrophobic amino acids (28, 29) and propensity to form amphipathic helices. Residues 74–99 of AKAP 79 fulfill both criteria (Fig. 2). Interestingly, this sequence, Thr-Arg-Gly-Ala-Trp-Ala-Ser-

Leu-Lys-Arg-Leu-Val-**Thr**-Arg-Arg-Lys-Arg-**Ser**-Glu-**Ser**-Ser-Lys-Gln-Gln, contains three of the four potential PKA phosphorylation sites present in AKAP 79 (Fig. 2). Chimeric proteins that include this region of AKAP 79 fused to a carrier molecule, endonexin II, are phosphorylated by PKA although endonexin is not.[2] Future studies are planned to determine if this region represents the calmodulin-binding site and if PKA phosphorylation alters binding. Phosphorylation by protein kinase C of similar sites in the MARCKS protein and neuromodulin prevents calmodulin binding (30–32).

Biochemical and immunohistochemical studies have demonstrated that approximately 75% of the type II PKA in the central nervous system is compartmentalized through interaction of its regulatory subunit and specific anchoring proteins (33–35). In particular, RII binds to neurons with high affinity and is believed to be attached to the dendrites through association with MAP 2 (6, 35, 36). However, RII has also been detected in other neuronal compartments and presumably is localized through association with different RII-anchoring proteins (37). Supporting this view, the immunological evidence presented in this study suggests that AKAP 79 and AKAP 150 are components of the human and murine postsynaptic densities. These data suggest that AKAP 79 and AKAP 150 function to anchor the type II PKA to the PSD through high affinity interaction with RII. Consistent with this hypothesis are data showing that approximately 30% of the total protein kinase activity, assayed using Kemptide as substrate, in purified PSD fractions was due to PKA (Fig. 8). Furthermore, this activity is attributed to the type II PKA since only RII was detected in purified PSD fractions by Western blot (Figs. 7*C* and 8). The remaining 70% of kinase activity in the PSD is likely due to CaM-kinase II. CaM-kinase II, which constitutes approximately 30% of total PSD protein, is capable of phosphorylating Kemptide (19).

A variety of data has indicated that protein phosphorylation regulates the efficiency of synaptic transmission, both by modulating the release of neurotransmitter from the presynaptic terminals and by modulating the sensitivity of receptors in the postsynaptic membrane (38). PSDs are a disc-shaped fibrous network of proteins located on the internal surface of excitatory synapses just inside the postsynaptic membrane (20). Protein kinases associated with the PSD are optimally positioned to respond to signals transduced across the synaptic membrane resulting from synaptic transmission or neurotransmitter release. Accordingly, all three multifunctional protein kinases, PKA, protein kinase C, and the Ca^{2+}/calmodulin-dependent protein kinase II have been detected in the PSD (20, 39–42). Two classes of PSD exist that have different morphologies and presumed functions (43). The PSD from type I synapses is a prominent band of fibrous material found in neurons that mediate excitatory responses. In contrast, PSD from the type II synapse is present in neurons that mediate inhibitory responses and is less pronounced with a punctate morphology. Previous studies have detected PKA associated with the type I PSD but not with the type II PSD (20). Additionally, several of the type I PSD proteins are *in vitro* substrates for PKA (20). This is consistent with the function of AKAP 79 and AKAP 150 to localize PKA in the type I PSD where it can regulate aspects of excitatory synaptic transmission. Presumably, AKAP 79 acts as a protein bridge where the amino-terminal domain associates with other PSD proteins, including calmodulin, and the carboxyl-terminal domain binds the PKA holoenzyme through interaction with RII.

Compartmentalization of PKA close to the postsynaptic membrane, through interaction with AKAP 79 and 150, places the kinase close to the neurotransmitter receptors. These receptors are particularly appropriate targets for modulation by PKA phosphorylation since they are central to the process of signal transduction across the postsynaptic membrane (38). Reports have shown that members of both classes of neurotransmitter receptors, G-protein-linked receptors and chemically gated ion channels, are phosphorylated by PKA (44, 45). In both cases, the functional effect of phosphorylation appears to be the regulation of receptor desensitization for their agonists. The β-adrenergic receptor binds the neurotransmitter norepinephrine or the hormone epinephrine and is a substrate for three different protein kinases, including PKA (46). Heterologous desensitization of the β-adrenergic receptor is controlled by PKA phosphorylation and reduces the ability of the receptor to activate G_s (47). Receptors for the excitatory amino acids α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-kainate and glutamate are potentiated by agents that stimulate PKA activity (48, 49). It is of interest to note that the glutamate receptor, R6 isoform (GluR6), contains a consensus PKA phosphorylation site in the cytoplasmic tail (50). At this time it is unknown whether GluR6 activity is regulated by cAMP. However, it is tempting to speculate that AKAP 79 and 150 adapt PKA for a function in synaptic plasticity by localizing the kinase close to specific substrates such as the neurotransmitter and excitatory amino acid receptor channels.

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REFERENCES

1. Scott, J. D. (1991) *Pharmacol & Ther.* **50,** 123–145
2. Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975) *J. Biol. Chem.* **250,** 7795–7801
3. Corbin, J. D., Keely, S. L., and Park, C. R. (1975) *J. Biol. Chem.* **250,** 218–225
4. Sarkar, D., Erlichman, J., and Rubin, C. S. (1984) *J. Biol. Chem.* **259,** 9840–9846
5. Lohmann, S. M., DeCamilli, P., Einig, I., and Walter, U. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6723–6727
6. Threukauf, W., and Vallee, R. (1982) *J. Biol. Chem.* **257,** 3284–3290
7. Rubino, H. M., Dammerman, M., Shafit-Zagardo, B., and Erlichman, J. (1989) *Neuron* **3,** 631–638
8. Obar, R. A., Dingus, J., Bayley, H., and Vallee, R. B (1989) *Neuron* **3,** 639–645
9. Leiser, M., Rubin, C. S., and Erlichman, J. (1986) *J. Biol. Chem.* **261,** 1904–1908
10. Bregman, D. B., Bhattacharyya, N., and Rubin, C. S. (1989) *J. Biol. Chem.* **264,** 4648–4656
11. Bregman, D. B., Hirsch, A. H., and Rubin, C. S. (1991) *J. Biol. Chem.* **266,** 7207–7213
12. Hirsch, A. H., Glantz, S. B., Li, Y., You, Y., and Rubin, C. S. (1992) *J. Biol. Chem.* **267,** 2131–2134
13. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. S., Brennan, R.G., and Scott, J. D. (1991) *J. Biol. Chem.* **266,** 14188–14192
14. Carr, D. W., Hausken, Z., Fraser, I. D. C., Stofko-Hahn, R. E., and Scott, J. D. (1992) *J. Biol. Chem.* **267,** 13376–13382
15. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
16. Stofko-Hahn, R. E., Carr, D. W., and Scott, J. D. (1992) *FEBS Lett.* **302,** 274–278
17. Studier, F. W., Rosenberg, A. H., Dubendorff, J. W., and Dunn, J. J. (1990) *Methods Enzymol.* **185,** 60–89
18. Nelson, R. M., and Long, G. L. (1989) *Anal. Biochem.* **180,** 147–151
19. Rich, D. P., Colbran, R. J., Schworer, C. M., and Soderling, T. R. (1989) *J. Neurochem.* **53,** 807–816
20. Carlin, R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. (1980) *J. Cell Biol.* **86,** 831–843
21. Corbin, J. D., and Reimann, E. M. (1974) *Methods Enzymol.* **38,** 287–294
22. Scott, J. D., Stofko, R. E., MacDonald, J. R., Comer, J. D., Vitalis, E. A., and Mangeli, J. (1990) *J. Biol. Chem.* **265,** 21561–22566
23. Scott, J. D., Fischer, E. H. Demaille, J. G., and Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4379–4383
24. Scott, J. D., Glaccum, M. B., Fischer, E. H., and Krebs, E. G. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1613–1616

[2] J. D. Scott and R. E. Stofko-Hahn, unpublished observation.

25. Towbin, H., Staehlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
26. Parker, C. W. (1990) *Methods Enzymol.* **182,** 721–737
27. Kozak, M. (1991) *J. Biol. Chem.* **266,** 19867–19870
28. Bagchi, I. C., Huang, Q., and Means, A. R. (1991) *J. Biol. Chem.* **267,** 3024–3029
29. Erickson-Vitanens, S., O'Neil, D. T., and DeGrado, W. F. (1987) in *Protein Engineering* (Oxender, D. L., and For, D. F., eds) pp. 201–211, Alan R. Liss, Inc., New York
30. McIllroy, G. K., Walters, J. D., Blackshear, P. J., and Johnson, J. D. (1991) *J. Biol. Chem.* **266,** 4959–4964
31. Graff, J. M., Rajan, R. R., Randall, R. R., Nairn, A. C., and Blackshear, P. J. (1991) *J. Biol. Chem.* **266,** 14390–14398
32. Chapman, E. R., Au, D., Alexander, K. A., Nicolson, T. A., and Storm, D. R. (1991) *J. Biol. Chem.* **266,** 207–213
33. Lohmann, S. M., Walter, U., and Greengard, P. (1980) *J. Biol. Chem.* **255,** 9985–9992
34. Miller, P., Walter, U., Theurkauf, W. E., Vallee, R. B., and De Camilli, P. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 5562–5566
35. Erlichman, J., Sarkar, D., Fleischer, N., and Rubin, C. S. (1980) *J. Biol. Chem.* **255,** 8179–8184
36. Vallee, R. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 3206–3210
37. Ludvig, N., Ribak, C. E., Scott, J. D., and Rubin, C. S. (1990) *Brain Res.* **520,** 90–120
38. Huganir, R. L., and Greengard, P. (1990) *Neuron* **5,** 555–567
39. Grab, D. J., Berzins, K., Cohen, R. S., and Siekevitz, P. (1979) *J. Biol. Chem.* **254,** 8690–8696
40. Grab, D. J., Carlin, R. K., and Siekevitz, P. (1981) *Ann. N. Y. Acad. Sci.* **33,** 51–57
41. Ueda, T., Greengard, P., Berzins, K., Cohen, R. S., Blomberg, F., Grab, D. J., and Siekevitz, P. (1979) *J. Cell Biol.* **83,** 308–319
42. Uno, I., Ueda, T., and Greengard, P. (1976) *J. Biol. Chem.* **252,** 5164–5174
43. Gray, E. G. (1959) *J. Anat.* **93,** 420–433
44. Hemmings, H. C., Nairn, A. C., McGuinness, T. L., Huganir, R. L., and Greengard, P. (1989) *FASEB J.* **3,** 1583–1592
45. DeCamilli, P., Bentenati, F., Valtorta, F., and Greengard, P. (1990) *Annu. Rev. Cell Biol.* **6,** 433–460
46. Benovic, J. L., Bouvier, M., Caron, M. G., and Lefkowitz, R. J. (1988) *Annu. Rev. Cell Biol.* **4,** 405–428
47. Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) *J. Biol. Chem.* **264,** 12657–12665
48. Yang Wang, L., Slater, M. W., and MacDonald, J. F. (1991) *Science* **253,** 1132–1135
49. Greengard, P., Jen, J., Nairn, A. C., and Stevens, C. F. (1991) *Science* **253,** 1135–1138
50. Nakanishi, N., Schneider, N. A., and Axel, R. (1990) *Neuron* **5,** 569–581